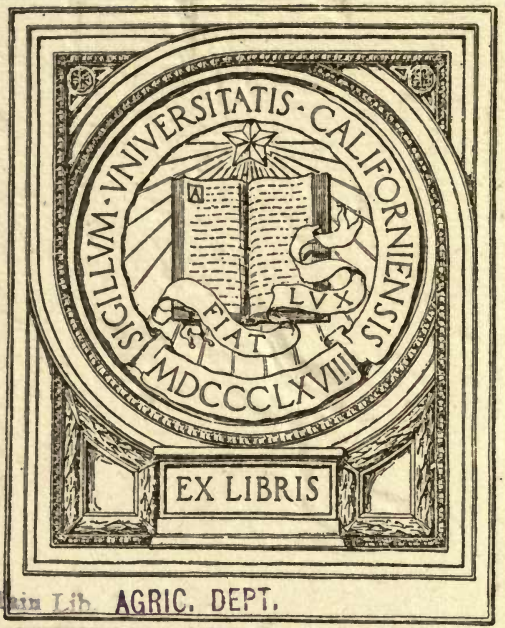

LABORATORY DIRECTIONS

FOR

BEGINNERS IN BACTERIOLOGY

BY

VERANUS A. MOORE

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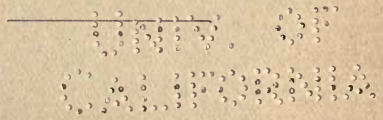

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PREFATORY NOTE.

It has been found desirable to provide the student, just beginning the study of bacteriology, with a somewhat detailed outline of the work to be done at each laboratory session. The selecting of the particular things to be done and the choosing of methods to be followed are difficult tasks. The assigning of directions for doing work under assumed conditions must necessarily partake of the empirical, and often fail. It is evident, however, that practical bacteriology must, if successfully taught, be cast in a somewhat definite form in order that the student may come to a knowledge of the fundamental principles underlying the subject in its twofold capacity, that of a pure science and of a useful art.

These outlines are intended to serve simply as a guide through an introductory laboratory course preparatory either to independent research work, or to form the basis for the application of the principles of bacteriology in the practice of human or of comparative medicine. They aim to impart a technical and working knowledge of certain of the more essential methods and to develop a definite knowledge of a few important species of bacteria. During the past year, they were furnished the students in mimeographed sheets, but after making the changes suggested by this application it seems desirable to put them in a more convenient form. In adjusting the amount of work for each exercise to the necessary limitations of time and facilities, I am indebted to Mr. Raymond C. Reed, Instructor in this Department, for much valuable assistance. I wish also to thank Prof. Charles Wright Dodge of the University of Rochester for helpful suggestions. Should these outlines fall in the hands of other teachers or workers in this subject, criticisms are cordially invited.

V. A. M.

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Text-book upon the Pathogenic Bacteria. By Joseph M'Farland.
A Text-book of Bacteriology and Infective Diseases. By Edward M. Crookshank.
Manual of Bacteriology. By Muir and Ritchie.
Micro-organisms and Disease. By E. Klein.
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Journal of Pathology and Bacteriology.
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Annales de l'Institut Pasteur.
Archives de Med. Experimental et d'anat. Pathologique.
See also standard Medical and Veterinary journals.

LABORATORY MAXIMS.

1. See that the working table, instruments, and all pieces of apparatus used are thoroughly cleaned at the close of each exercise.

2. Unless otherwise directed all cultures, other than those in gelatin, are to be grown in the incubator.

3. Gelatin cultures should not be put in the incubator.

4. In opening tubes of media or cultures, always flame the open end of the tube immediately after withdrawing the plug. If the tubes have been standing for some time the surface of the plug should be flamed before drawing it out. Never allow the tube end of the plug to touch, while out of the tube, any article by which it could become contaminated. It should be held by the top between the fingers.

5. In every case where a platinum wire loop or needle is used for making cultures or withdrawing media it should be carefully heated in a gas flame both immediately before and after using. The heated wire must be allowed to cool before making cultures.

6. If by accident, a drop or more of a culture should be spilled upon the table or floor, pour over it a sufficient quantity of a disinfectant (corrosive sublimate solution 1–1000, or a 5% solution of carbolic acid) to completely cover the affected area. After this has acted for ten minutes wipe it up and boil or burn the cotton or cloth. If any of the culture should drop on the hands or clothing a disinfectant should be applied immediately.

7. In sterilizing culture media, always see that there is enough water in the pan of the steam sterilizer or in the water bath before lighting the gas. Do not put the media in a sterilizer and leave the laboratory.

8. Always disinfect, by boiling, all cultures before cleaning the tubes or plates containing them.

9. At the beginning of each laboratory session read the directions for the next exercise in order to be able to make any preliminary preparations which may be required.

APPARATUS AND MATERIAL.

A. *For general use.* This includes the apparatus and chemicals to be used in common by all students. It consists of pans and brushes for cleaning test tubes and other glass ware, meat mincer and press, large and small water baths, steam sterilizers, hot-air sterilizers, incubators, thermometers, thermostats, gas burners, balances, leveling tripods, Wolffhügels' or other apparatus for aids in counting colonies, micrometers, metric rules, burettes, tripods, funnels, beakers, pipettes, graduates, glass tubing and rods. The chemicals necessary for carrying on the work, such as various acids and alkalies, disinfectants, alcohol, aniline dyes, and those articles needed in the preparation of culture media, such as salt, peptone, agar, gelatin, meat extract, sugars, litmus and other indicators, and filter paper. Fresh meat, eggs, milk and potatoes must be furnished as needed. It also includes color charts and the more important books of reference.

B. *For individual use.* These are various appliances used by each student and for which he becomes personally responsible. They consist of a microscope with substage condenser, two oculars (1 and 2 in.) and three objectives ($\frac{2}{3}$, $\frac{1}{6}$ and $\frac{1}{12}$ inch), a bottle of immersion oil, a tripod magnifier, 75 small test tubes, 50 large test tubes, 12 fermentation tubes, 18 Petri dishes, 3 Erlenmeyer flasks, 7 one ounce bottles for reagents and stains, 7 pipettes with rubber bulbs to fit bottles, 1 platinum-wire loop, 1 platinum-wire needle, 3 tin cups for holding cultures, 3 tin boxes for holding test tubes, 1 block for holding reagent bottles, 1 glass slide with ring attached for hanging-drop preparations, 1 tin tray for cover-glass preparations, 4 solid watch glasses, 2 Stender dishes for used slides and cover-glasses, and a glass box for clean cover-glasses.

Each working table is provided with a reserve-flame, gas burner (Bunsen), glass jars for waste, and stands for holding culture tubes. A requisite amount of absorbent cotton, lens paper, and towels are furnished.

Each student is to provide himself with a box of slides and cover-glasses ($\frac{3}{4}$ inch square cover-glasses preferred; they must be between .12 and .18 mm. in thickness), gummed labels for slides and tubes, a Faber's blue pencil for marking on glass, fine forceps for handling cover-glasses, and paper for laboratory notes with manilla card board covers.

EXERCISE I.

CLEANING GLASSWARE.

§ 1. **Explanatory note.** It is necessary that the glassware employed should be thoroughly cleaned before it is used. Several special methods have been suggested for this purpose but the one frequently employed by chemists seems to be the most easily handled and quite as efficient for general use as the more elaborate, specialized processes. It consists in applying the chromic acid cleaning mixture after washing the tubes and flasks with water. It is sometimes necessary to employ other methods for cover-glasses which are to be used in staining bacteria where a mordant is required. But one of these special methods will be given here.

§ 2. **General directions.** Clean all of the glassware, test tubes, fermentation tubes, flasks, Petri dishes and reagent bottles assigned.

Put the slides and cover-glasses in the cleaning mixture, they can be rinsed and wiped later.

Careful notes should be taken on the work of each exercise. These should be handed to the instructor for examination and correction as often as every two weeks.

(*a*) *Test tubes.* Wash these carefully with soap and water using the test tube brush. After washing stand them in a glass jar (aquarium) and fill them to within about 2 cm. of the top with cleaning mixture (this will be kept in stock in the laboratory). After it has acted for from 5 to 10 minutes (it is better to stand longer) pour it out of the tubes into the bottle originally containing it. Rinse the tubes thoroughly in tap water until all color disappears and then in hot water and drain them, using individual drainage

board from locker. After they are dry, wipe the outside of the tubes with a slightly dampened cloth.

(*b*) *Fermentation tubes.* Treat these in the same manner as the test tubes excepting in the use of the brush which must be omitted.

(*c*) *Flasks.* Wash the flasks thoroughly with soap and water. Then fill them with the cleaning mixture and allow it to act for at least 10 minutes after which it can be poured back. Rinse the flasks thoroughly in the same manner as the test tubes and drain them. When dry the outside should be wiped with a damp cloth.

(*d*) *Petri dishes and reagent bottles.* Thoroughly wash the Petri dishes and reagent bottles in hot soap suds after which rinse them separately in hot water. Wipe the Petri dishes dry with a cloth and drain the bottles. The cleaning mixture need not be used.

(*e*) *Slides and cover-glasses.* Drop the cover-glasses singly into a glass jar containing cleaning mixture and allow them to remain there for 24 hours or longer. Pour off the cleaning mixture and rinse the cover-glasses until all of the color disappears, then cover them with alcohol until needed when they can be wiped with a soft linen cloth or with lens paper. Treat the slides in the same manner as the cover-glasses. They can be wiped directly out of the rinsing water. The slides can be cleaned satisfactorily by washing them in a strong, hot soap suds, rinse in water and wipe.

(*f*) *Cleaning used culture apparatus.* Place the tubes, flasks, or Petri dishes containing old cultures in a water bath, cover them with water to which add a little sal soda (about an ounce to the gallon of water) and boil for 20 minutes. Pour off the water and empty the tubes after which again boil them for 5 minutes in clean soap and water. Then wash and treat with the cleaning mixture the same as the new tubes.

§ 3. **Formula for chromic acid cleaning mixture.** Dissolve 80 grams of potassium dichromate ($K_2Cr_2O_7$) in 300 c.c. of warm water, when all of the $K_2Cr_2O_7$ is dissolved and the solution cooled add it slowly, with constant stirring, to 460 c.c. concentrated sulphuric acid with constant stirring, store the mixture in a glass-stoppered bottle.

§ 4. **A method for cleaning cover-glasses for flagella stain.** For this work the ordinary method of cleaning cover-glasses is not sufficient. The following treatment was recommended to me by Dr. Erwin F. Smith. After being cleaned by the ordinary method boil the cover-glasses in an agate cup or glass beaker in a 10% solution of caustic soda for five minutes. After cooling rinse the cover-glasses thoroughly in distilled water after which place them in a beaker and cover with a 1% solution of hydrochloric acid, heat to the boiling point, and allow to stand for several hours (over night or longer). Then pour the acid off and rinse the cover-glasses several times in distilled water and finally in alcohol. Wipe them out of alcohol as they are needed.

EXERCISE II.

PLUGGING THE TUBES AND FLASKS AND STERILIZING THE GLASSWARE.

§ 5. **Explanatory note.** After the tubes and flasks are cleaned they must be plugged. The plugged tubes and flasks and Petri dishes, all of which are to be used for holding culture media or in making cultures, must be sterilized before they can be used. The plugs should be neatly made and of the proper length and firmness. Absorbent cotton is ordinarily used for this purpose, although common cotton is employed in some laboratories. Glassware is sterilized by means of dry heat, *i. e.*, in the hot air sterilizer. (See methods for sterilizing apparatus and instruments in text-books.)

§ 6. **General Directions.** Plug all of the tubes and flasks with absorbent cotton and sterilize them, together with the Petri dishes, in the hot air sterilizer. After they are sterilized store them in the locker until they are needed. The Petri dishes must not be opened until they are used.

§ 7. **Plugging the tubes and flasks.** The rolls of absorbent cotton are cut in short segments of from 5 to 7 cm. in length. A piece of sufficient length of this narrow strip to give cotton enough for the plug is torn off. The quantity varies, of course, with the size of the mouth of the tube, or flask, but a little experience will enable one to estimate the quantity accurately. The torn edges of the cotton are turned in and it is rolled up to form a firm plug which should snugly fit the neck of the tube or flask. It should be inserted into the tube for about 2 cm. and the end should be nearly flat and smooth. The projecting part should be about the same length and be of equal firmness.

§ 8. **Sterilizing glass ware.** Place all of the tubes, flasks, and Petri dishes in the hot air sterilizer, close the door tightly and light the gas. Heat the air in the sterilizer to a temperature of from 135 to 150° C. and keep it there for one hour, (the temperature must not be allowed to go above 150° C.) Then turn the gas off and when the temperature of the air in the sterilizer goes down to or below 45° C. the door can be opened and the apparatus removed.

EXERCISE III.

THE PREPARATION OF BOUILLON.

§ 9. **Explanatory note.** Bouillon is the liquid medium most commonly employed in cultivating bacteria. It is practically a beef tea containing peptone. There are several methods recommended for making it. (1) it may be made directly from simple meat infusion or, (2) it may be made from meat extract. The meat infusion is prepared either by

allowing finely chopped lean meat mixed with twice its quantity of water (2 c.c. of water for each gram of meat) to stand in a cool place for from 12 to 18 hours, or, the mixture of meat and water may be heated with frequent stirring at a temperature of 65° C. for a short time (one hour). Each method has its peculiar advantages. When the meat is macerated at a low temperature the muscle sugar is frequently lost. When it is made by heating the meat the bouillon usually contains a trace of muscle sugar. When meat extract is used in place of the meat infusion the bouillon does not seem to be a favorable culture fluid for certain bacteria. In making bouillon therefore it becomes necessary to determine the kind (whether from meat infusion or extract) and the method of preparing it to suit the conditions in hand. It is sometimes desirable in bacteriological investigations to resort to all of these methods. For the routine work in the laboratory, bouillon prepared directly from the meat by macerating it at a high temperature (65° C.) is very satisfactory.

Bouillon is used as the nutritive base in preparing agar and gelatin. On this account the large quantities are stored in flasks. (For other methods see text-books. Also Journal of the American Public Health Asso., Jan. 1898, p. 77.)

§ 10. **General Directions.** Make 1000 c. c. of bouillon and distribute it as follows:

Put 7 c. c. in each of 10 small sterile test tubes.

Put 300 c. c. in each of 2 flasks and the balance in a small flask.

Put 7 c. c. of distilled water in each of 5 small sterile test tubes and sterilize them with the bouillon. These tubes should be steamed or boiled for 10 minutes on each of the two succeeding days. (They are to be used subsequently in place of bouillon in making dilutions). Label the tubes, "Sterile distilled water."

Note. All media, in addition to that made by the student,

required to carry out the directions for the fall term will be furnished.

§ 11. **The preparation of bouillon.** Take 500 grams of lean beef, remove all fat, and grind it in a sausage machine or have it minced at the butcher shop. Place the minced meat in an agate iron dish and add 1000 c. c. of distilled or boiled water, (2 c. c. for each gram of meat) and thoroughly stir with a glass rod. Then macerate it with frequent stirring in a water bath at a temperature of 65° C. for 1 hour after the temperature of the meat and water reaches that of the water outside. Remove the meat by straining the liquid through a piece of cheese cloth. For this a meat press is desirable. The liquid should equal in quantity the amount of water used, if it does not, add distilled or boiling water to make it up to that amount. To this meat infusion add ¼ per cent. peptone (Witte's) and ½ per cent. sodium chloride. Add enough of a 1% solution of sodium hydrate to give the liquid a faintly alkaline reaction. In this work the alkalinity can be determined by the use of sensitive litmus paper. (For neutralizing culture media for special or research work, see Jour. Am. Pub. H. A., Jan. 1898, p. 73.) The infusion is then boiled in a water bath for ¾ of an hour, and allowed to cool. When cool filter it through ordinary filter paper. The filtrate should be perfectly clear. The color will vary according to the amount of blood pigment in the meat used. After filtering, distribute the bouillon in tubes and flasks. See above. Stand the tubes containing the bouillon in a wire basket for sterilization. Sterilize them by boiling in a closed water bath or steaming in the Arnold's steam sterilizer for 30 minutes,* the time to be

*The customary method of sterilizing culture media is to steam or boil it for about 10 minutes on each of three consecutive days. This was found very troublesome by the students and feeling that it was not necessary a long series of test experiments were made by Mr. R. C. Reed, who found that one boiling or steaming for 30 minutes gave

computed from the time the water boils. The flasks of bouillon should be boiled or steamed for 20 minutes on each of the two succeeding days. When they have cooled, the outside of the tubes should be carefully wiped with a moist cloth and placed in the incubator until the next laboratory day. Then carefully examine them and if any of the tubes are contaminated, that is, if the liquid is clouded or has a membrane on the surface, they must be rejected. Label all others and place them in the locker.

§ 12. **Labeling.** Stick on each tube of media, about 3 cm. from the top, an adhesive, white label about 2 cm. square. On the upper lines should be written the name of the medium, and the date of its preparation. Thus, Bouillon, 13–VII–97. When the tube is used the name of the organism or material with which it is inoculated, together with the date of inoculation, should be written on the lower lines. This applies to all media and cultures.

EXERCISE IV.

THE PREPARATION OF AGAR AND GELATIN.

§ 13. **Explanatory note.** Of the solid media employed in cultivating bacteria, agar and gelatin are most commonly used. They depend for their nutritive properties largely upon the bouillon from which they are made, the agar and gelatin forming simply the solidifying elements. The striking difference between the two is that the gelatin melts at the body temperature whereas the agar is not liquefied below the boiling point. For this reason gelatin cannot be used as

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just as good results as the customary 3 boilings. As the media is not used for 2 or 3 days after its sterilization and during which time it is kept in an incubator, the method is adopted, not that it saves time in preparing the media, but it relieves the congestion in the sterilizer and appreciably aids the student.

a solid medium for cultivating bacteria at a high (body) temperature. There are several processes for preparing these media but the addition of the dry agar and gelatin to bouillon (§ 11) either immediately after it is filtered or later after it has been sterilized and stored in flasks seems to be the most convenient procedure. The agar itself is usually neutral in reaction but the gelatin often has a decidedly acid reaction. This necessitates the careful testing of the reaction of the two media.

§ 14. **General directions.** Prepare 300 c.c. of agar and 300 c.c. of gelatin, *i. e.*, start with 300 c.c. of bouillon for each. There will be considerable shrinkage so that the quantities of media will be appreciably less than this amount. Distribute each medium as follows :

Put 7 c.c. in each of 10 small sterile test tubes.
Put 12 c.c. in each of 12 large sterile test tubes.
Put the balance in a small sterile flask.

§ 15. **Nutrient gelatin.** Take a flask of bouillon containing 300 c.c. and pour it into a small agate iron dish and add 30 grams of sheet gelatin, which has been cut into small pieces, heat the bouillon, with frequent stirring, in a water bath until the gelatin is dissolved. Allow it to cool to a temperature between 45° and 50° C. and then add the white of one egg and mix it thoroughly by stirring or better by pouring the gelatin from one flask or beaker to another. After the egg albumen is completely dissolved return the liquid gelatin to the large covered water bath and boil until the egg albumen is coagulated. This takes about 20 minutes. It is now ready for filtering which must be done while the gelatin is hot. Filter through properly folded but ordinary filter paper, first moistened with boiling water. (For illustrations and directions for folding filter paper see Abbott's Prin. of Bact. p. 82). Distribute the filtrate as directed. In pouring the gelatin into the tube use a small

beaker or graduate and see that the gelatin does not touch the sides of the upper part of the tube. Stand the tubes in a wire basket and sterilize them by boiling in a closed water bath or by steaming in the Arnold's steam sterilizer for 30 minutes. The small flasks can be sterilized in the same manner. Place tubes and small flasks in the incubator and allow them to remain there for two days. If the gelatin in any of the tubes becomes cloudy the medium in those tubes must be rejected. Carefully wipe all of the other tubes with a moist cloth, label and place them in the locker where they can be kept until used.

§ 16. **Nutrient agar.** Weigh out 3 grams of agar and cut it into small pieces with a pair of scissors. Put the finely cut agar into an agate iron dish and add 50 c.c. of distilled water and boil it over a gas flame with constant stirring, to prevent scorching, until the agar is dissolved, giving a thick homogeneous pasty substance. Pour 300 c.c. of bouillon (§ 10) from a flask into an agate iron cup and to it add the dissolved agar. Place the dish containing the mixed agar and bouillon in a closed water bath and boil for 20 minutes. Then cool it to a temperature between 45 and 50° C. and add the white of one egg and thoroughly mix it in the liquid agar. This is easily accomplished by pouring it from one beaker to another. When the egg albumen is dissolved the agar is returned to the water bath and boiled vigorously until the white of the egg is coagulated. This usually takes about 20 minutes. Filter the agar immediately, while hot, through ordinary filter paper which has been moistened with boiling water. Distribute the filtrate in small and large tubes, as directed. Sterilize, label, and store the agar in the same manner as the gelatin.

EXERCISE V.

INOCULATING TUBES OF BOUILLON, AGAR AND GELATIN.

§ 17. **General directions.** Inoculate a tube of bouillon, two (one inclined the other not) of agar and one of gelatin from a culture of *Bacillus coli communis* which will be furnished.

Wipe out the slides and transfer the cover-glasses to the alcohol (§ 2 e).

Read the chapters in the text-books on inoculating media or making tube cultures.

§ 18. **Inoculating bouillon.** In making this culture carefully remove the plug from the tube of bouillon by first twisting it around to detach any adhesions and then pull it straight out. Pass the open end of the tube quickly through the gas flame. The plug, which has meantime been carefully held, is partially replaced and the tube returned to its stand. Treat the tube containing the culture (which has been furnished) in the same manner. Then place the two tubes side by side between the thumb and fore finger of the left hand and grasp them about the middle of the upper half. Sterilize the platinum loop by passing it through the gas flame, care being taken that the handle is flamed for a distance of at least 15 cm. Then carefully remove the plugs from the tubes and hold them between the fingers in such a manner that the tube ends will not touch anything during the inoculation process. Insert the wire loop carefully into the culture and transfer a loopful of the culture to the tube of bouillon and gently rinse it from the loop. The loop is then withdrawn, the plugs replaced in their respective tubes and the loop flamed and put aside. Label the freshly inoculated tube with the name of the organism, source and date. Stand it in a tray or cup and place it in the incubator. This should be kept at a temper-

ature between 35 and 37°C. The organism thus transferred should multiply so that on the following day the liquid will be cloudy. This is a bouillon culture of *B. coli communis.*

§ 19. **Inoculating tubes of agar.** Ordinarily the agar is inclined before it is inoculated. In this case it is spoken of as inclined or slant agar. Occasionally the agar is inoculated without inclining it. Cultures made in this manner are spoken of as "stab" or "stick" cultures. (1) Inclined or slant agar. Stand a tube of agar in a wire basket in a water bath and boil it until the agar is liquefied. (To save repeating this it is well to incline the agar in several tubes which can be kept for future use). Lay the tubes on a tray, the top resting on the side of the tray so that the surface of the agar will be about 4 cm. long, allow it to cool. In placing the tubes the label should be up. When the agar has set it is ready for use. It is inoculated precisely as the bouillon excepting the loopful of culture is drawn over the inclined surface instead of being thrust into the medium as in the bouillon. Label and place it in the incubator with the inoculated bouillon tube. On the following day there should be a grayish growth on the surface of the agar covered by the loop. This is an agar culture of *B. coli communis.* (2) Stick cultures. These are made with a platinum needle in the uninclined agar. The impregnated needle is pushed down through the center of the agar. In all other respects this culture is made like the slant agar culture.

§ 20. **Inoculating the tube of gelatin.** Tube cultures in gelatin are usually made without inclining the gelatin, *i.e.*, stick cultures. The tube of gelatin is inoculated in the same manner as the stick culture in agar. This tube is to be placed in the locker as the gelatin will melt at the incubator temperature. The growth will appear in about two days along the needle track. This is a gelatin culture of *B. coli communis.*

EXERCISE VI.

THE EXAMINATION OF CULTURES.

§ 21. **Explanatory note.** In studying cultures of bacteria, it is necessary to observe very carefully (1) the macroscopic appearance of the growth in or upon the media, (2) the microscopic appearance of the bacteria in (a) the living condition (hanging-drop preparation), and (b) in the dead and stained condition (cover-glass preparation), and (3) the effect of the growth of the bacteria upon the chemical and the physical properties of the medium. To determine these, the cultures must be kept under observation for several days and often weeks. A careful record should be made of the changes observed in the appearance of the cultures. Illustrate with drawings.

§ 22. **General directions.** Examine carefully and describe fully the appearance of the bouillon, agar, and gelatin cultures made in Exercise V.

Determine the reaction of the bouillon culture and note whether there is any change in its consistence (viscidity).

Make a hanging drop preparation from each culture and examine and describe the appearance of the bacteria in each.

Make a drawing of the gelatin and slant agar cultures and also of a few of the bacteria in one of the hanging-drop preparations.

Read the paragraphs in the text books on the examination of cultures and hanging drop preparations.

§ 23. **Suggestions for the macroscopic examination.** The external appearance of cultures should be observed and noted on the day after they are made and on each succeeding day until the growth ceases. In bouillon cultures the appearance of the liquid, whether uniformly, faintly or heavily clouded, turbid, clear or clouded with flocculent masses held in suspension, the quantity, and nature of sedi-

ment, and the presence or absence of a membrane should be noted. The reaction of the liquid, its consistence and odor should be determined. In the agar cultures the extent of the growth (feeble, moderate or vigorous,) its color, form and surface appearance (dull or glistening) should be observed. The character of the growth in the condensation water should also be noted. In stab cultures the appearance of the growth both on the surface and along the needle track should be described. In gelatin, the absence or the presence and extent of liquefaction should be noted in addition to the features already referred to for the stab agar cultures.

§ 24. **Testing the reaction of liquid cultures.** Place a small piece of each of the red and blue litmus paper in a solid watch glass. With the platinum loop carefully place a drop of the culture on each piece of the paper. After recording the reaction produced, neutral, acid or alkaline with the degree, cover the paper with a disinfectant (a solution of corrosive sublimate 1 to 1000). After it has acted for about 10 minutes empty it with the paper into the waste jar and wash the watch glass.

To determine the viscidity. (1) Bouillon cultures. Insert the platinum loop into the liquid and carefully withdraw it. The approximate degree of viscidity can be determined by the extent of the adhesion of the liquid to the loop, and the length of the thread-like filament drawn out. By gently shaking the tube a viscid sediment will rise up appearing as a somewhat twisted tenacious cone with its apex reaching to or near the surface. A friable sediment will break up and become disseminated through the liquid upon agitation. (2) Agar and gelatin cultures. Touch the surface growth with the end of the platinum needle and if it is viscid a thread-like string will be drawn out. Note whether the growth is pasty or friable.

§ 25. **Making hanging-drop preparations.** (1) From bouillon culture. Place a clean cover-glass on the tray.

With the loop, remove a drop of the liquid culture and place it on the middle of the cover-glass. With a pair of fine forceps invert the cover-glass over the glass ring fixed to a slide for this purpose. The surface of the ring should previously be moistened with liquid vaseline to prevent the cover-glass from sliding. The preparation is then ready for examination. Examine it first with the high power dry lens and then with the oil immersion objective. (For directions in the use of the microscope see "The Microscope" by Professor S. H. Gage.) (2) From cultures on solid media. On account of the very large number of bacteria in the growth on solid media it is necessary to separate them in a clear liquid. Take a cover-glass as before and place a loopful of sterilized water or bouillon on the center. With the platinum needle touch the surface growth very gently with the end of the needle and carefully rinse it in the drop of liquid on the cover-glass. From this point the examination is the same as with the liquid culture. Upon examination, if the bacteria are so numerous that the individual organisms can not be clearly distinguished, *i.e.*, separated from each other, the preparation must be rejected and another one made, using a smaller quantity of the growth.

§ 26. **Suggestions for the microscopic examination of bacteria.** In examining the bacteria, as they appear under the microscope in the hanging drop preparations, the following features should be observed: Are the individual bacteria spherical, rod-shaped or spiral in form? Are they single or united in pairs, masses or clumps, or in shorter or longer chains? For this determination it is better to examine the edge of the drop. Are they motile, that is, do the individual bacteria move from one point in the field to another? To determine this the center of the drop is better. Clearly distinguish between motility and a simple dancing motion. Determine the presence or absence of spores. These are bright highly refractory bodies either within or outside of

the bodies of the bacteria. If present they can usually be seen in both positions. Is there any evidence of a capsule around the bacteria?

EXERCISE VII.

MAKING AND STAINING COVER-GLASS PREPARATIONS, AND FORMULAE FOR STAINING SOLUTIONS.

§ 27. **General Directions.** Make two cover-glass preparations from each of the cultures made in Exercise V and stain one with alkaline methylene-blue and the other with carbol fuchsin. Describe the appearance of the bacteria and make a drawing of a few individual bacteria from the preparations made from the agar culture.

Preserve a cover-glass preparation mounted in balsam and labeled to accompany notes.

Prepare the staining fluids used in this exercise from the formulae given.

Read the paragraphs in the text-books on making and staining cover-glass preparations.

§ 28. **Making cover-glass preparations.** (1) From bouillon cultures. Place 2 clean cover-glasses on the tray. With the loop remove a drop of the bouillon culture and spread it in a thin layer over about ⅔ of the surface of the cover-glasses. One loopful will ordinarily make from 2 to 4 preparations. Allow the liquid to dry on the cover-glasses in the air. When dry fix the bacteria to the cover-glasses by passing them, film upward, 3 times through the middle of the upper half of the gas flame. Each passage (complete circle) should not occupy more than one second. After fixing they are ready for staining (§ 29). (2) From cultures on solid media (agar, gelatin, potato, serum, etc.). Place the cover-glasses on the tray and on the center of each a

drop of sterile water or bouillon. With the needle touch the surface growth of the culture and then gently rinse the end of the needle in the liquid on the covers. Spread the liquid on the covers as before. From this point the procedure is the same as that for the preparations made from the bouillon culture.

§ 29. **Staining bacteria in cover-glass preparations.** (1) With alkaline methylene-blue. With the pipette place a few drops of the staining solution on the film side of the preparation which is either held horizontally with the fine forceps or left resting on the tray. Allow the stain to act for 2 or 3 minutes. Then carefully rinse off the stain in water, holding the cover firmly by one edge with the forceps. After thoroughly rinsing place the preparation, film downward, on a clean slide and dry the upper surface with a piece of filter paper. It is now ready for the microscopic examination. Use first the dry lens (⅛ in. obj.) and then the oil immersion objective. If the specimen is a good one and it is desirable to preserve it wipe off the drop of oil with a piece of lens paper, run a drop of distilled water under the cover-glass which floats it, when it can be easily removed with the forceps. Place it on the tray, film upward, and when dry mount it in alkaline Canada balsam. (2) With carbol-fuchsin. Cover the film on the cover-glass with the stain and allow it to act for about one minute. Then rinse it thoroughly in water after which cover it with $\frac{1}{10}\%$ solution of acetic acid or strong (95 %) alcohol. Allow this to act from 5 to 10 seconds and again thoroughly rinse in water and examine as above. (For other decolorizers, see text-books.)

Upon examination the preparation should be free from deposits or stained back ground. The bacteria should, as a rule, be isolated and distinct, unless they are, the preparations are not satisfactory.

Cover-glass preparations of bacteria are permanently

mounted in the same manner as similar preparations made from the blood or other tissues in histology, the process being to place a drop of the balsam on the center of the slide and place the preparation, film downward, over it and apply slight pressure. Label the preparation, giving the name of the organism, its source, (kind of culture, tissue, etc., from which the preparation was made), stain used, and date. If the specimen is not preserved the slide and cover-glass should be cleaned for future use.

§ 30. **Suggestions concerning the microscopic examination of stained preparations of bacteria.** In the examination of the bacteria in the stained condition the following points, and perhaps, others should be observed and noted. (1) Concerning their morphology. Are they spherical, rod-shaped, or spiral? Are they separated or united in clumps or chains? If rod-shaped, are the ends pointed, round, or square? Are the bacteria all of the same form and size? Note the presence or absence of spores and capsules. (2) Concerning their reaction to staining fluids. Do they stain uniformly or irregularly? Do they stain deeply or faintly? Is the center lighter than the periphery? Is there an unstained central band and deeply stained ends (polar stain)? Do all of the bacteria take the stain alike?

§ 31. **Staining solutions.** The basic aniline dyes are used in staining bacteria. There is a large number of these, and there are several formulae for preparing staining solutions from each. Further, as will be seen from the chapters on staining bacteria in the text-books, there are several methods of applying these stains. In an introductory course, however, it is impossible to try them all and consequently those are described which seem to be the best adapted for general use.

In addition to the ordinary staining solutions and methods there are special processes for certain species such, for example, as the tubercle bacillus, and still others for stain-

ing certain parts of many bacteria, such as the flagella on motile forms, the spores in spore bearing organisms, and the capsule on certain other species. There is a large number of these special methods but in this course only one of each will be given. These will be taken up in connection with the study of the bacteria requiring them.

§ 32. **Formulae for staining solutions.** The dyes used are methylene-blue, gentian-violet, methyl-violet, and fuchsin.

LOEFFLER'S ALKALINE METHYLENE-BLUE.

Concentrated alcoholic solution of methylene-blue	30 c.c.
Caustic potash 1 per cent. solution	1 c.c.
Distilled water	100 c.c.

CARBOL FUCHSIN (ZIEHL'S SOLUTION).

Fuchsin (dry)	1 gram.
Alcohol (Absolute)	10 c.c.
Carbolic acid, 5 per cent. solution	100 c.c.

Dissolve the fuchsin in the alcohol, after which add the carbolic acid solution. Instead of using the dry fuchsin and alcohol, 10 c.c. of a saturated alcoholic solution of fuchsin may be used.

§ 33. **Aqueous Solutions.** Aqueous solutions of methyl-violet, gentian-violet, fuchsin, and the other aniline dyes are prepared by adding 1 c.c. of the saturated alcoholic solution of the desired dye to 20 c.c. of distilled water. This will impart a decided color to the liquid so that a pipette full will be barely transparent.

The true aqueous solutions are made by dissolving the dyes in water, but these are weak and not so effective as those prepared from the alcoholic solutions. These solutions deteriorate in a short time. The carbol fuchsin and alkaline methylene-blue will keep a little longer, but they require to be filtered occasionally.

ANILINE METHYL-VIOLET (EHRLICH-WEIGERT).

Saturated alcoholic solution of methyl-violet	11 c.c.
Absolute alcohol	10 c.c.
Aniline water	100 c.c.

§ 34. **Making aniline water.** Aniline water is a saturated aqueous solution of aniline oil. It is prepared by adding 1 c.c. of aniline oil to 20 c.c. of distilled water and shaking frequently for from 15 to 30 minutes. It is convenient to use a stoppered vial or large test tube for mixing it. Filter through a moistened filter paper. The filtrate should be perfectly clear. If it is cloudy it should be refiltered before using.

EXERCISE VIII.

MAKING PLATE AND ESMARCH ROLL CULTURES.

§ 35. **Explanatory note.** The general principle underlying the separation of bacteria by means of plate and roll cultures is to dilute the substance containing the bacteria so that the individual organisms will be separated from each other by an appreciable distance and then fixed in a solid medium where each organism can multiply into a growth or colony without coming in contact with any other organism or colony. For this purpose agar and gelatin are used. Originally, Koch employed a rectangular piece of glass for holding the layer of medium and protected it from contamination by putting it under a bell jar. Later Esmarch introduced the "roll culture" method which was extensively followed, until the Petri dishes were introduced. Since that time they have been largely used in place of the Koch plate and Esmarch tube. On this account the plate cultures of to-day are usually made in Petri dishes. The roll culture, however, is occasionally made.

§ 36. **General Directions.** Make a series of 3 agar plates, one of 3 gelatin plates, and a series of 3 gelatin roll cultures (Esmarch rolls) from the bouillon culture of *Bacillus coli communis* (§ 18). Place the agar plates in the incubator and the gelatin plates and rolls in a locker for that purpose.

Re-examine all the cultures made in previous exercises and add to the laboratory notes a description of any changes in their appearance. The notes should contain a detailed record of the cultures made in this exercise.

Read carefully the paragraphs in the text books on making plate and roll cultures.

§ 37. **Making agar plates.** Take three large tubes of agar, stand them in a water bath and boil until the agar is liquefied. Then cool by standing the tubes with a thermometer in a cup of water at a temperature of about 50° C. As the temperature rises add a little cold water. When the temperature of the agar reaches that of the water and the temperature of the whole has lowered to 45° C. the agar is ready for use. For convenience in labeling number the tubes 1, 2, and 3.

Place 3 sterilized Petri dishes on the leveling tripod and adjust it by means of a spirit level. With the wire loop proceed by the same method as followed in making bouillon cultures. Take one loopful of the bouillon culture and place it in agar tube No. 1 and mix by carefully shaking it. Flame the wire and transfer two loopfuls of agar from tube 1 to tube 2 and mix as before. Again flame the loop and transfer 3 loopfuls from tube 2 to tube 3 and mix as with tubes 1 and 2. After the tubes are inoculated, pour the agar into the Petri dishes. In doing this remove the plug, flame the mouth of the tube, and after quickly cooling, raise with the left hand the edge of the cover on one side of the dish sufficiently to allow of inserting the mouth of the tube, and hold it until the agar is poured and replace it immediately. Label, and number the Petri dishes to correspond

with the dilutions in the tubes, thus, plate 1 is from tube one, plate 2 is from tube 2, and plate 3 is from tube 3. In making the dilutions it is important that the wire loop should be flamed after making each transfer.

§ 38. **Making gelatin plates.** These are prepared precisely as the agar plates with these exceptions. (1) The gelatin is liquefied at a temperature of 45° C. (2) The plates when made are to be kept in the locker the same as the gelatin stab cultures. (3) In hot weather it is sometimes necessary to put a piece of ice in the reservoir under the glass plate on the leveling tripod to congeal the gelatin.

The directions given above for making the dilutions are applicable only when the original culture is moderately clouded. If there are comparatively very few bacteria in the liquid a larger quantity of the culture will be necessary. If there are many more, as in turbid bouillon or slant agar culture, it will be necessary to take a much smaller quantity for the first dilution. It is often desirable to make the first dilution in a tube of sterile water or bouillon instead of gelatin or agar, and to make two rather than three plates.

§ 39. **Making Esmarch roll cultures.** For this purpose gelatin is ordinarily used as agar does not adhere readily to the sides of the tubes. It is sometimes used. Take the desired number of large tubes of gelatin, liquefy, inoculate, label and number the dilutions as in making gelatin plate cultures. Place a block of ice about 6 inches long in an agate iron tray. Melt a slight horizontal groove in the ice with a test tube containing hot media or water. The inoculated tubes are tipped and rolled so that the liquid gelatin moistens the inside of the tube to within about a centimeter of the plug. Then roll the tube rapidly in the groove on the ice until the medium becomes solid. The gelatin should not come in contact with the plug. In rolling the tube the plugged end should always project beyond the ice. See illustration in text books.

EXERCISE IX.

THE EXAMINATION OF PLATE CULTURES AND MAKING SUBCULTURES FROM COLONIES.

§ 40. **Explanatory note.** In practical bacteriological work plate cultures are made use of in determining (1) the number of bacteria there is in a given substance; (2) the different species of bacteria present, and (3) the character of the growth in a colony of the organism in question. Other important facts, such, for example, as the relative number of each species of bacteria, or the difference in the appearance of the surface and deep colonies are learned through this process. The plate culture, therefore, is one of the most important single methods employed in isolating and studying bacteria.

§ 41. **General directions.** Examine carefully and describe the plate cultures made in Exercise VIII. If the agar plates do not have colonies, or if the colonies are so numerous that they cannot be counted on any of the plates, make the cultures over again, and give an explanation in the notes of this exercise for the failure to obtain good results.

Make a hanging drop preparation from a colony from an agar plate, and one from a colony from a gelatin plate, and examine them microscopically. Describe the appearance of the bacteria in each.

Make a cover-glass preparation from the same colonies and stain each with carbol fuchsin. Examine each carefully and make a drawing of a few of the isolated bacteria. Describe (§ 30) the appearance of the bacteria in these preparations.

Inoculate a tube of bouillon, one of agar and one of gelatin from a well isolated colony from one of the agar plates.

§ 42. **Suggestions for the examination of the plate and roll cultures.** Observe the general appearance of the

plates, note whether the colonies are well isolated or run together (confluent); describe the appearance of the individual colonies, (a) those on the surface, (b) those in the depth of the medium. Indicate their shape (round, lenticular, flat, convex or spherical). Are the edges sharply defined? Is the margin even or irregular? Give their size (diameter in millimeters), and indicate their color (determine shade from a color chart), and consistence. Do the surface colonies adhere to the medium or can they be easily removed? Examine them with a low lens and describe the surface markings if any. Also indicate the difference in color as observed with the unaided eye and with the microscope.

§ 43. **Estimating the number of colonies on plates.** If the number of colonies is not large (not to exceed 100) they can be counted and the exact number recorded. This may be done with the third plate. When the number is larger it is more convenient to divide the total area into smaller ones and count the number of colonies in each of several (20 to 40) of the small areas, add these together and divide the sum by the number of areas counted. The quotient gives the average number on one area; multiply this quotient by the number of areas containing colonies and the product will be the number of colonies on the plate. This latter process, however, gives the approximate number only.

For dividing the area of the plate into smaller, equal areas, it is convenient to use Wolffhügel's counting apparatus. This was devised more particularly for square or oblong plates (Koch). In counting the colonies on the Petri dishes Parker's scheme modified by Jeffers is more suitable. It consists of a disc about 20 cm. in diameter divided into areas of a square centimeter each. Place the Petri dish over the disc taking care that it is accurately centered.

Count the number of colonies in several (20 to 40) of the areas and multiply the mean number by the number of areas

covered. This product gives the approximate number of colonies.

§ 44. **Making subcultures from colonies.** Select the tubes of media to be used, and flame the mouths as heretofore described. Select a colony as well isolated from all others as possible. With the left hand carefully raise the edge of one side of the cover of the Petri dish, and while holding it, touch the colony with the needle, replace the cover, take up the tube of media and inoculate it. If bouillon is used first a tube of agar or gelatin can be inoculated immediately afterwards without recharging the needle. If more cultures are to be made it is necessary to again charge the needle from the colony. If the plate is to be rejected the cover can be entirely removed in the beginning. The newly inoculated tubes or subcultures should be labeled and treated according to the directions heretofore given for handling cultures. These inoculated tubes should be pure cultures. It sometimes happens, however, that what appears to be a single colony consists of the growth of two organisms. If these should be of different species the cultures made from the colony would probably be impure. These impure growths (apparently single colonies) frequently develop on plate cultures exposed to the air for some time. The particles of dust often carry two or more bacteria.

EXERCISE X.

THE PREPARATION OF CERTAIN SPECIAL MEDIA.

§ 45. **Explanatory note.** In studying the properties of bacteria it is desirable to cultivate them on a number of different media. Bouillon, agar, and gelatin are most commonly used, but others are necessary in determining the cultural peculiarities and important biochemic properties of the organism in question. The cultivation of bacteria upon

these media may be regarded somewhat as a test, to determine the presence or absence of certain properties. Thus, for example, will the species in hand coagulate the casein in milk, produce gas in media containing saccharose, grow on potato, etc.? The number of these tests which have been used and recognized as important is quite large, but in a short course only those possessed of the more differential value can be tried. In describing a new species, or identifying any of the carefully described ones, it is important to know at least some of these cultural peculiarities and biochemic properties. For this reason it is necessary to learn the method of preparation and the use of certain of these media.

In addition to the above, a few species of bacteria require a particular kind or kinds of media for their diagnostic or most differential growth.

Among these are those of glanders, diphtheria and tuberculosis. The preparation of these particular media will be considered in connection with the study of the organisms requiring them.

§ 46. **General Directions.** Prepare for culture media, 5 tubes of potatoes, 5 tubes of milk, 5 tubes of litmus milk, 5 tubes of glucose agar, 5 tubes of glycerin agar, 4 fermentation tubes of bouillon containing glucose, 4 containing lactose, and 4 containing saccharose. (The agar and the sugar free bouillon necessary in the work of this exercise will be furnished by the instructor.)

Read carefully the paragraphs in the text books on the preparation and use of these media.

§ 47. **Preparation of potato for a culture medium.** Select medium sized potatoes, thoroughly wash and cut out, with a cutter made for this purpose, a cylinder 3 to 4 cm. long (oblong pieces cut with a knife will do quite as well). Ordinarily 2 cylinders can be cut from each potato. The inclined surface is obtained by cutting out the potato pro-

jecting above the frame of the cylindrical knife. All of the skin must be removed. Wash the potato cylinders in cold, running water for some 5 minutes (a longer time is preferable) and place them in test tubes of the proper size (large or small according to size of cutter used), and add about 1 c.c. of water to each tube. Sterilize them by discontinuous boiling or steaming for 20 minutes each day for three consecutive days. Wipe, label, and store in locker.

§ 48. **Preparation of milk for a culture medium.** Place about 100 c.c. of fresh milk in a beaker in the ice box and allow it to stand for from 10 to 15 hours. Then carefully remove the cream. It is well to filter the milk through a thin layer of absorbent cotton to remove any masses of cream. The reaction should be tested and if strongly acid it should be rejected or made 1.5% acid to phenolphthaleni by the addition of n/1 sodium hydrate. Distribute the skimmed milk in small test tubes (7 c.c. in each) and sterilize by discontinuous steaming in the same manner and for the same length of time as the potatoes. Label and store in locker.

§ 49. **Preparation of litmus milk for a culture medium.** This is prepared the same as the milk medium with the addition of enough of an aqueous solution of litmus to impart a decidedly blue color to the milk. Sterilize label and store the same as the milk. The litmus solution will be furnished.

§ 50. **Preparation of glucose agar.** Prepare 100 c.c. of agar (§ 16). Reserve one half of it for glycerine agar and to the other half add 1% glucose. Dissolve the powdered glucose in about 5 c.c. of boiled, hot water before adding it to the liquid agar. After thoroughly mixing distribute it in small sterile test tubes. Sterilize, label, and store the same as ordinary agar.

§ 51. **Preparation of glycerine agar.** Take the balance of the agar prepared above (§ 50) and add 5% of pure glycerin. Thoroughly mix it with the liquid agar, after which distribute it in tubes. Sterilize, label, and store as ordinary agar.

§ 52. **Preparation of glucose bouillon.** This is used in the fermentation tube. Take 100 c.c. of peptonized bouillon (§ 11) and add 1 gram of pure grape sugar (glucose). After it is dissolved and thoroughly disseminated through the bouillon by stirring or pouring, distribute the bouillon in the fermentation tubes, filling completely the closed branch and the open bulb about half full. Sterilize it by discontinuous steaming for 20 minutes each day for three consecutive days. It should be labeled and kept in the locker until needed for use.

§ 53. **Preparation of lactose bouillon.** This is prepared by adding 1% lactose to the peptonized bouillon. It is necessary, however, that the bouillon used does not contain muscle sugar. Bouillon free from muscle sugar can usually be obtained by macerating the meat for from 12 to 18 hours (§ 9) at a low temperature. After adding the lactose and thoroughly mixing it in the bouillon, sterilize, label, and store the same as the glucose bouillon.

§ 54. **Saccharose bouillon.** This is peptonized bouillon to which 1% saccharose has been added. It is prepared from bouillon free from muscle sugar, in the same manner as lactose bouillon.

EXERCISE XI.

INOCULATING SPECIAL MEDIA AND EXAMINING CULTURES.

§ 55. **General Directions.** Inoculate a tube of potato, one of milk, one of litmus milk, one of glucose agar, a fermentation tube of glucose, one of lactose and one of saccharose bouillon. Label each and place all of them in the incubator.

Examine microscopically the agar and bouillon cultures made from the colony on the agar plate (§ 41). Examine and carefully describe the bacteria from each culture in, (1)

a hanging-drop preparation, and (2) stained cover-glass preparations. Stain a preparation with alkaline methylene-blue, one with carbol fuchsin, and one with an aqueous solution of methyl-violet. Make a careful comparison of the three preparations and note any difference in the appearance of the bacteria or in the degree of intensity of the stain. Preserve as a permanent specimen, to accompany the notes, a preparation stained with each of the dyes.

Prepare the aqueous solution of methyl-violet (§ 33).

§ 56. **The inoculation of glucose agar to determine the power of the organism to produce gas.** Boil the tube of glucose agar in an open water bath until it is liquefied, then cool it down to a temperature of 45° C. and inoculate it with a loopful of the culture, carefully stir the agar with the loop, after which solidify the agar as quickly as possible. Label and stand in the incubator.

EXERCISE XII.

THE EXAMINATION OF CULTURES ON SPECIAL MEDIA.

§ 57. **Explanatory note.** As certain of these media are used to determine the effect of the bacteria upon them it is important to observe very carefully not only the appearance of the growth of the bacteria, but also their effect, if any, upon the medium upon or in which they are growing. This is especially noticeable in the milk, litmus milk and sugar bouillon cultures. The changes here are largely due to the action of the bacteria on the sugars or their power to produce alkali.

§ 58. **General Directions.** Examine and describe the cultures made on the special media in Exercise XI.

Examine the bacteria on the potato culture microscopically (1) in the fresh condition (hanging-drop preparation), and

(2) in stained cover-glass preparations. Stain a preparation with carbol fuchsin and one with an aqueous solution of gentian-violet. Describe the appearance of the bacteria (§ 33) and make a drawing of a few of them.

§ 59. **A few points to be observed in studying cultures on special media.** (*a*) *Potato.* Note carefully the extent and color of the growth and also its consistence.

(*b*) *Milk.* Note whether or not the general appearance and odor of the milk has been changed, observe whether the casein has been coagulated giving a firm solid coagulum, or precipitated. Is the coagulum covered with a liquid (serum), if so, is it clear or milky? Is there any appearance suggestive of saponification. Determine its consistence, chemical reaction as indicated by litmus paper (§ 24), and give as descriptive a name as possible to its odor.

(*c*) *Litmus milk.* Note especially whether there has been any change in color since inoculation. Observations similar to those on the plain milk should also be made.

(*d*) *Glucose agar.* Note the character and number of colonies within the agar, and the presence, if any, of gas bubbles. Are there few or many of them?

(*e*) *Glucose bouillon.* Observe the character of the growth (whether the liquid is faintly or heavily clouded, turbid, contains flakes, etc.) in (1) the open branch and (2) the closed branch of the fermentation tube. Note the presence or absence of a membrane on the surface of the liquid in the open bulb. Is there a sediment in the bottom of the tube? If so, describe its general appearance and consistence. Note the presence or absence of gas in the closed branch. Indicate the quantity. Test the reaction of the liquid with litmus paper.

(*f*) *Lactose and saccharose bouillon.* Same as glucose bouillon. These cultures should be observed from day to day and note made of any changes which have occurred.

The fermentation tubes are used to enable one to determine the quantity and kinds of gases produced and also the anaërobic properties of the organism.

EXERCISE XIII.

THE EXAMINATION OF CULTURES (continued).

§ 60. **General Directions.** Re-examine the cultures made on special media and make notes on all changes which have occurred in their appearance.

Examine microscopically in hanging-drop preparations the bacteria from the glucose-bouillon culture.

Make 3 stained cover-glass preparations from the milk culture. Stain with the different dyes already used.

Re-examine all of the cultures previously made and make careful notes of any changes in appearance.

Reject all of the cultures made excepting those on agar from the colony which should be preserved and clean the tubes and Petri dishes (§ 2 f.).

§ 61. **Making cover-glass preparations from milk cultures.** Spread as thin a film as possible of the milk culture on the cover-glass and allow it to dry in the air. Then pass it 3 times through a flame. After flaming immerse the preparation in a watch glass, or other receptacle, containing a few cubic centimeters of ether to dissolve out the fat. Then remove and after the ether has evaporated stain as usual. The amount of albumen in the milk will usually cause a heavy background which will require decolorizing with alcohol or weak acetic acid.

EXERCISE XIV.

THE CLASSIFICATION OF BACTERIA.

§ 62. **Explanatory note.** Bacteria is a general and popular term used to designate a large group of microscopic plants, the *Schizomycetes*. These organisms which are widely distributed in nature have been classified into a certain few families and genera most of which have a large number of species. Many of these species have been described, but there are many which have not. In classifying the bacteria the genera are based on morphologic characters while, as a rule the species are determined by means of their biochemic, physiologic, or pathogenic properties. Several systems of classification have been proposed but the one which seems to be the most satisfactory is by Migula. This classification utilizes the morphology to such good advantage that its adoption seems desirable. It requires, however, some serious changes in the accustomed nomenclature. The restoration of the genus Bacterium, and the assigning to it of all non-motile, rod-shaped organisms changes the genus of some of our most common pathogenic bacteria from Bacillus to Bacterium. The most conspicuous of these are the Bacilli of tuberculosis, glanders, and diphtheria, all of which are placed in Migula's classification in the genus Bacterium. The families and genera recognized by him are appended.

FAMILIES.

I. Cells globose in a free state, not elongating in any direction before division into 1, 2 or 3 planes ..... 1. Coccaceae.

II. Cells cylindrical, longer or shorter, and only dividing in one plane, and elongating to twice the normal length before the division.

(1) Cells straight, rod-shaped, without sheath, non-motile, or motile by means of flagella ..... 2. Bacteriaceae.

(2) Cells crooked, without sheath_____3. Spirillaceae.
(3) Cells enclosed in a sheath_____4. Chlamydobacteriaceae.
(4) Cells destitute of a sheath, united into threads, motile by means of an undulating membrane_____5. Beggiatoaceae.

GENERA.

1. Coccaceae.

Cells without organs of motion.
a. Division in one plane_____1. Streptococcus.
b. Division in two planes_____2. Micrococcus.
c. Division in three planes_____3. Sarcina.
Cells with organs of motion.
a. Division in two planes_____4. Planococcus.
b. Division in three planes_____5. Planosarcina.

2. Bacteriaceae.

Cells without organs of motion_____1. Bacterium.
Cells with organs of motion (flagella).
a. Flagella distributed over the whole body_____2. Bacillus.
b. Flagella polar_____3. Pseudomonas.

3. Spirillaceae.

Cells rigid, not snake-like or flexuous.
a. Cells without organs of motion_____1. Spirosoma.
b. Cells with organs of motion (flagella).
1. Cells with 1, very rarely 2–3 polar flagella_____2. Microspira.
2. Cells with polar flagella-tufts____3. Spirillum.
Cells flexuous_____4. Spirochaeta.

4. Chlamydobacteriaceae.

Cell contents without granules of sulphur.
a. Cell threads unbranched.
I. Cell division always only in one plane__1. Streptothrix.
II. Cell division in three planes previous to the formation of conidia.
1. Cells surrounded by a very delicate, scarcely visible sheath (marine)_____2. Phragmidiothrix.
2. Sheath clearly visible (in fresh water)_3. Crenothrix.
b. Cell threads branched_____4. Cladothrix.
Cell contents containing sulphur granules_5. Thiothrix.

5. Beggiatoaceae.

Only one genus known (*Beggiatoa* Trev.) which is scarcely separable from *Oscillaria*. Character as given under the family.

Of the genera the Streptococcus, Micrococcus, Bacterium, Bacillus, Microspira and Spirillum contain the most important of the pathogenic bacteria. The familiar genus Staphylococcus of an older classification is included in the genus Micrococcus by Migula. It is important that the distinguishing characters of these genera are thoroughly learned.

§ 63. **General directions.** Read the references* on the morphology and classification of bacteria.

Learn from the text-books and lecture notes the more essential elements in the structure of bacteria.

Inoculate a tube of bouillon from a culture which will be furnished of each of the following bacteria and place the inoculated tubes in the incubator.

(1) A streptococcus_____(Streptococcus _____).
(2) A micrococcus_____(Micrococcus _____).
(3) A staphylococcus_____(Staphylococcus____).
(4) A sarcina_____(Sarcina lutea_____).
(5) A non-motile rod-shaped organism_(Bacterium ____ ____).
(6) A motile, rod-shaped organism____(Bacillus _____ ____).
(7) A spiral, rod-shaped organism____( ).

EXERCISE XV.

IDENTIFYING GENERA AMONG BACTERIA.

§ 64. **General directions.** Carefully describe each of the bouillon cultures made in Exercise XIV.

Prepare and examine a hanging drop preparation from each of the cultures, and describe the appearance (form) of

*Migula, Die Naturlichen Pflanzenfamilien, Lieferung 129, Leipzic, 1896. Migula, System der Bakterien, 1897. Fischer, Jahrbücher für wissenschaftliche Botanik, Band XXVII, Erstes Heft.

the organisms in each. Indicate the morphologic characters by which each genus can be differentiated from the others.

Make a cover-glass preparation from each culture and stain with an aqueous solution of Methyl-violet. Make a careful microscopic examination of each preparation and describe the bacteria in each.

Make careful notes on the appearance of the bacteria in each preparation and preserve one specimen of each to accompany notes.

Measure carefully with the filar micrometer the length and thickness of three individual bacteria in the stained preparation of the bacillus. Record the measurements in microns. (For the use of the micrometer see chapter on magnification and micrometry in "The Microscope" by Professor S. H. Gage.)

Inoculate a tube of agar and one of gelatin from each of the bouillon cultures.

EXERCISE XVI.

STUDYING THE MORPHOLOGY OF BACTERIA.

§ 65. **General directions.** Examine and describe the cultures made in Exercise XV.

Examine in the fresh condition and in stained cover-glass preparations the bacteria from the agar cultures and make careful notes on their forms. Stain with alkaline methylene-blue and carbol fuchsin. Measure a few of the individual bacteria in the preparations of the micrococcus, and streptococcus.

Inoculate a tube of agar, one of gelatin and one of bouillon with *Bacillus subtilis* from a culture furnished and place the inoculated tubes in the incubator.

EXERCISE XVII.

STUDYING AND STAINING SPORES.

§ 66. **Explanatory note.** In certain species of bacteria and under suitable conditions, there appears within the bacteria highly refractory granules or bodies known as spores. The formation of spores is largely restricted to certain species of bacilli. The spores are oval in form and in old cultures they can often be found outside of the bodies of the organisms which produce them. They possess the power of resisting drying, heat, and unfavorable environment much longer than the bacilli themselves. Ordinarily they do not stain by the usual methods employed in staining bacteria so that special methods are required. Several processes have been proposed, but the one here given seems to be quite as efficient as any of the others.

Bacillus subtilis, or the hay bacillus, is one of the most widely distributed species of bacteria. It develops spores which can be readily detected in either the fresh or stained preparations from cultures.

§ 67. **General directions.** Examine and carefully describe the three cultures of *Bacillus subtilis*.

Make a hanging drop preparation from the bouillon and one from the agar cultures and examine them microscopically. Describe the bacilli and observe carefully the appearance of the spores both within and outside of the bacilli.

Make a cover-glass preparation from each culture and stain with alkaline methylene-blue. Examine carefully and note the appearance of spores which remain unstained. Make a drawing of a few of the bacteria containing spores.

Make a few (about 3) cover-glass preparations and stain them for spores.

Inoculate a tube of agar with *Bacillus cholerae suis* and place it in the incubator for the next exercise.

§ 68. **A method for staining spores.** Make a cover-glass preparation, dry, and flame as already described. Take the preparation by the edge with the fine forceps, cover the film surface with carbol fuchsin and hold the preparation over the gas flame until steam is given off, then remove it for a few seconds and again heat it. Repeat the heating 3 or 4 times. After the stain has acted for from 3 to 5 minutes rinse the preparation in water and decolorize it by immersing it in a watch-glass containing about 3 c.c. of a 1% solution of sulphuric acid or 95% alcohol. After about one-half minute remove the preparation and rinse it thoroughly in water. If it is not decolorized repeat the bleaching process. This removes the coloring matter from the bodies of the bacteria, but leaves it in the spores. After thoroughly washing the preparation, counter stain it with a saturated aqueous solution of methylene-blue for about 30 seconds, rinse in water and examine. The spores should be stained red (with the fuchsin) and the rest of the organism should be colored blue.

There is a very satisfactory method recommended by Möller. For this and other methods for staining spores, see text-books on bacteriology.

EXERCISE XVIII.

STAINING THE FLAGELLA ON MOTILE BACTERIA.

§ 69. **Explanatory note.** The motile bacteria are provided with a variable number of long hair-like appendages or flagella. These are invisible in the fresh preparation and they do not stain by the ordinary methods. By special staining processes, however, their presence can be detected. Several methods have been proposed for staining these filaments but nearly all of them are based on the use of a

mordant. Curiously enough the value of each of these methods seems to rest largely in the individual using them, as some workers succeed with one method while others fail with it but obtain excellent results with one of the other processes. Although the flagella are thought to be the organs of locomotion they do not seem to be of any special morphological value in differentiating closely related species. They are, however, elements in the structure of motile bacteria and their demonstration is much to be desired.

§ 70. **General directions.** Make a cover-glass preparation from the growth on the agar culture of *Bacillus cholerae suis* made in Exercise XVII and stain it with carbol fuchsin. Preserve this to compare with preparations stained for the purpose of demonstrating the flagella.

Clean about 20 cover-glasses after the special method for flagella staining (§ 4). Make about 10 cover-glass preparations on these from the agar culture and stain for flagella. Use Loeffler's method but if it does not succeed the second process may be tried.

§ 71. **Making cover-glass preparations for flagella stain.** Place 2 loopfuls of sterilized, distilled water on the center of the cover-glass. Gently touch the surface growth on the agar culture with the end of the platinum needle and immerse it in the water on the cover-glass without spreading the drop. The impregnated needle should carry bacteria enough for 3 or 4 preparations. Then place the tray of cover-glasses in the incubator to dry. The bacteria become disseminated throughout the water by means of their power of locomotion. When dry they are ready for the staining treatment.

§ 72. **Staining the flagella by Loeffler's method.** The bacteria are fixed to the cover-glasses by holding them, film upward, between the thumb and fore finger, over a gas flame

for about a minute. They are then treated with the following mordant :

Tannic acid, 20% solution	10 c.c.
Sulphate of iron, saturated solution	5 c.c.
Fuchsin, saturated alcoholic solution	1 c.c.

This should be filtered before using.

Place the fixed cover-glass preparation in a large test tube, cover it with the mordant and carefully heat over a gas flame or in a water bath until steam is given off. Allow the mordant to act for from 3 to 5 minutes. Then remove the cover-glass with a bent wire loop and fine forceps and thoroughly rinse it in water. Then place it in a similar tube and cover it with carbol-fuchsin for staining. Heat this as the mordant, and allow it to act for from 5 to 10 minutes. Remove the cover-glass as before and thoroughly rinse in water. If the stain is too deep decolorize by rinsing the preparation for a few seconds in alcohol and again in water. It is then ready for the microscopic examination either in water or it may be allowed to dry and be mounted in balsam. If the first preparation fails add 2 drops of a 10% solution of sulphuric acid to the mordant.

The flagella should appear as fine, hair-like appendages radiating from the bacteria.

§ 73. **Staining the flagella by Van Ermengem's method.** The films are prepared as described above. Three solutions are necessary :

Solution A. (Fixing bath).

Osmic acid, 2% solution	1 part.
Tannin 10–25% solution	2 parts.

Place the films in this for one hour at room temperature, or heat over a flame till steam rises, and keep in the hot stain for five minutes. Wash with distilled water, then with absolute alcohol for 3 to 4 minutes, and again in distilled water, and treat with Solution B.

Solution B. (sensitizing bath) This is a 5 per cent. solution of silver nitrate in distilled water. Allow the films to be in this for from 2 to 3 minutes. Then without washing transfer to Solution C.

Solution C. (reducing and strengthening bath).

Gallic acid	5 grams.
Tannin	3 grams.
Fused potassium acetate	10 grams.
Distilled water	350 c.c.

Keep in this for 1 to 1½ minutes. Wash, dry and mount. It will also be found an advantage to use a fresh supply of C for each preparation, a small quantity being sufficient.

EXERCISE XIX.

STAINING TUBERCLE BACTERIA (BACILLI).

§ 74. **Explanatory note.** The tubercle bacterium possesses the power of retaining when stained the coloring matter when treated with a strong decolorizer such as a solution of sulphuric or nitric acid. On this account its stain has a high differential value which is made use of in identifying this organism. Thus, in the examination of sputum in cases of suspected tuberculosis, the object is to determine the presence of tubercle bacteria. As this organism is not easily cultivated the staining process is very largely depended upon in making a diagnosis.

§ 75. **General Directions.** Make three cover-glass preparations from a culture of tubercle (furnished). Stain these and carefully describe the appearance of the bacteria and illustrate them with a few drawings.

Stain a cover-glass preparation of tubercular sputum (furnished).

Read the directions in the text-books for staining tubercle bacteria (bacilli).

§ 76. **Staining tubercle bacteria.** Prepare the cover-glass preparations from the culture or the tuberculous material and flame them as already described. Stain in fresh carbol fuchsin. Place a few drops of the stain on the film-side of the cover-glass and hold it over a flame with forceps until steam is given off. Allow the hot stain to act for from 3 to 5 minutes. Or, the preparation may be floated on the carbol fuchsin in a watch glass without heat. In this case it is allowed to act for from 10 to 15 minutes. The preparation is then rinsed in water, and decolorized by treating it with a 10% solution of nitric or sulphuric acid for ¼ to one minute. It is again rinsed in water when it is ready for examination. It can be dried and mounted permanently in balsam. The tubercle bacteria should be stained a deep reddish color. All other bacteria or animal tissue in the preparation should be unstained. If desired, a counter stain, such as alkaline methylene-blue can be used after decolorizing. That is, the preparation can be again stained for about one minute in alkaline methylene-blue, rinsed in water and examined as before. In these preparations the tubercle bacteria are red and the other organisms and cells are blue. A counter stain is of no value in preparations made from pure cultures or for simple diagnostic purposes. When a counter stain is desired Gabbett's decolorizing and counter staining solution is very convenient.

Formula :—

Methylene-blue (powder)	2 grams.
10 % Sulphuric acid	100 c. c.

(Gabbett recommended 25 % solution of sulphuric acid.)

After staining with the carbol fuchsin treat the preparations with this mixture until the film has a faintly bluish tint. This solution decolorizes and counter stains at the same time.

EXERCISE XX.

MAKING CULTURES OF ANAËROBIC BACTERIA.

§ 77. **Explanatory note.** Anaërobic bacteria will not grow in the presence of oxygen (atmosphere) and consequently they must be cultivated in a medium from which the air has been expelled, or in the presence of some neutral gas such as hydrogen. The known important pathogenic anaërobic bacteria are those of symptomatic anthrax, tetanus and malignant oedema. These are known as obligatory anaërobes because they require the absence of oxygen. Others such as *Bacillus typhosus* are known as facultative anaërobes as they will multiply in media with or without atmospheric oxygen.

There are several methods of cultivating anaërobic bacteria but as a rule they are difficult and can not be easily handled in a general course. Two of the simpler processes, however, will be tried.

§ 78. **General Directions.** Inoculate a tube of agar after Liborius' method for anaërobic bacteria from the culture furnished.

Inoculate 2 fermentation tubes from the same culture. One of the fermentation tubes should contain sugar free bouillon (furnished) the other bouillon containing 1% glucose.

Inoculate for study at the next Exercise 2 tubes of liquid agar, (one plain and one containing glucose), a fermentation tube of sugar free bouillon and one containing 1% glucose bouillon with *B. coli communis* from a culture furnished.

§ 79. **Liborius' method.** Liquefy two tubes of agar and carefully pour them together. After this boil the medium for at least five minutes to expel the air, cool it down to a temperature of 45° C. and then inoculate it from the cul-

tures of anaërobic bacillus furnished. (B.———) after which cool the medium rapidly until it is set. In inoculating the tube insert the loop nearly to the bottom and stir very gently. In making the inoculations care must be taken not to introduce air by shaking the liquid medium. Place the culture in the incubator.

§ 80. **The fermentation tubes for anaërobic bacteria.** If these tubes of bouillon have been properly sterilized the closed branch is practically free from atmosphere. The obligatory anaërobe will grow in the closed branch only, while the facultative anaërobe will grow in both the open and closed parts. If the organism is a gas producer, the gas will force the cloudy liquid from the closed bulb into the open one clouding the otherwise clear liquid. To avoid the possibility of error in interpreting these growths it is well to inoculate a tube containing sugar free bouillon in which case the liquid in the open bulb should remain clear as gas will not be formed.

These tubes are of equal value in testing obligatory and facultative anaërobic organisms.

EXERCISE XXI.

EXAMINATION OF ANAËROBIC CULTURES.

§ 81. **General directions.** Examine and carefully describe the appearance of the anaërobic cultures made in Exercise XX.

With the wire loop remove one of the colonies from the depth of the agar culture and examine it microscopically in (a) a hanging-drop preparation, and (b) a stained cover-glass preparation. Stain with carbol fuchsin. Examine microscopically in similar preparations the bacteria from one of the fermentation tubes. Describe the appearance of the

bacteria in each preparation and make a drawing of a few of them.

Note the appearance of the cultures inoculated for the study of the gas production.

Read carefully in the text-books the methods for cultivating anaërobic bacteria.

EXERCISE XXII.

STUDY OF THE GAS PRODUCTION BY BACTERIA.

§ 82. **Explanatory note.** The knowledge of the powers of a given species of bacteria to produce gas when grown in a medium containing sugar is quite important. It is desirable to determine both the quantity of gas and its relative composition. Chemical analyses have shown that, in all cases tested, the gas consists of a mixture of hydrogen (H) and carbonic acid gas (CO_2) with mere traces of other gases. It is importart to know also the quantity of gas produced with the various sugars especially glucose, lactose and saccharose. To determine simply whether an organism will produce gas it is only necessary to inoculate a tube of liquid agar containing the sugars with it, but if the quantity of gas is to be determined the fermentation tube is the most convenient apparatus to use. In some cases the gas formation is one of the most striking differential properties as will be seen in the study of hog-cholera and typhoid bacilli. (For a discussion of the gas production and use of the fermentation tube see article by Dr. Theobald Smith, Wilder Quarter-century book p. 187).

§ 83. **General directions.** Examine and describe the cultures in the glucose and plain agar inoculated in Exercise XX. Note the approximate size and number of gas bubbles in the glucose agar, and explain the cause of difference in the number of bubbles in the two agar cultures.

Examine the fermentation tubes and indicate the quantity of gas, and the ratio of gas to liquid in the closed branch.

Determine the ratio of CO_2 to H in the gas.

In studying these cultures they should be examined each day and the quantity of gas indicated. Note the bubbles of gas rising through the liquid to the top. When the gas production has ceased the liquid begins to clear near the surface in the closed branch. The final record should not be made until this occurs. The reaction of the culture should be determined and noted.

Inoculate a tube of bouillon and make a series of three agar plates for Exercise XXIII from a mixed or impure culture, which will be furnished.

§ 84. **Determine the quantity of gas.** It is desirable to determine the quantity of gas collected in the closed branch in terms of the capacity of the tube. To do this measure the length of the closed branch and the length of that portion of the tube filled with gas. Thus if the length of the tube is 10 cm. and the length of the portion filled with gas is 3 cm. the gas fills $\frac{3}{10}$ of the branch. This can not be determined until the gas formation has ceased which sometimes requires several (4 to 6) days.

§ 85. **Determine the ratio of the CO_2 to H in the gas produced.** This can be approximately determined by the use of caustic soda. Remove the plug from the fermentation tube and fill the open bulb with a 2% solution of caustic soda. Place the thumb tightly over the open end of the tube and tip it up so that the gas will pass through the liquid and come into the open bulb. It is then returned. This should be repeated several times. Remove the thumb when the open bulb is full and the liquid will rush up into the closed branch to fill the space occupied by the CO_2 which has been absorbed by the caustic soda. Measure the portion of the tube first occupied with gas and now filled with the liquid. This will indicate the quantity of CO_2. The bal-

ance of the gas is H. (There are also traces of other gases). Its explosive property can be tested by filling the open bulb with water, cover it with the thumb and again bring the gas to the open bulb, hold it close to a flame and remove the thumb. A distinct explosion will be heard.

The ratio of CO_2 to H can be determined from the measurements. Thus the total amount of gas = 5 cm. The amount absorbed (CO_2) = 2 cm. The remaining gas or 3 cm. = the H. The ratio of CO_2 to H is, therefore, as 2:3 or CO_2:H: :2:3.

EXERCISE XXIII.

IDENTIFYING GENERA OF BACTERIA AND OBTAINING PURE CULTURES FROM COLONIES.

§ 86. **General directions.** Examine the bouillon cultures made from the impure or mixed culture in both the fresh condition and in stained cover-glass preparations. Stain with carbol fuchsin and an aqueous solution of methyl-violet. Describe the appearance of the bacteria in these preparations and note the number of genera present. Give the names of the genera and note the one which predominates in numbers.

Examine and carefully describe the colonies on the plate cultures. Determine the number of different colonies, and carefully describe each.

Inoculate a tube of bouillon from a colony from each genus. Label the inoculated tube with the name of the genus. Make stained cover-glass preparations from a colony of each genus Mount and label one of these preparations to accompany the notes.

EXERCISE XXIV.

CLEANING USED CULTURE TUBES, FLASKS, AND PETRI DISHES.

§ 87. **General directions.** Describe the bouillon cultures made from the colonies on the agar plates Exercise XXIII. Describe the bacteria in the stained cover-glass preparations and compare them with the bacteria in the preparations made from the colonies.

Reject and clean all tubes and Petri dishes that have been used for cultures.

Complete the notes on all work which has been done during the fall term.

Have all apparatus for individual use inspected by the instructor and released.

EXERCISE XXV.

PREPARATION OF GLASSWARE FOR CULTURE MEDIA.

§ 88. **Explanatory note.** It is desirable that the technique in plugging and sterilizing test tubes and flasks and in making culture media should be thoroughly understood. On this account this part of the work of the fall term is repeated, but hereafter in the course, the janitor will clean all glassware and the instructor will furnish all additional media required. It will be necessary in the following exercises to overlap to a considerable extent by way of inoculating media and in making the final examinations of cultures.

§ 89. **General directions.** Plug and sterilize the test tubes and flasks and sterilize the Petri dishes.

EXERCISE XXVI.

PREPARATION OF CULTURE MEDIA.

§ 90. **General directions.** Prepare, after the methods already given (Exercise III, IV and X) the following media :

15 small test tubes of bouillon.
10 small and 15 large test tubes of agar.
10 small and 15 large test tubes of gelatin.
5 small test tubes of glucose agar.
5 small test tubes of milk.
5 small test tubes of litmus milk.
5 small or large test tubes of potato.
5 small test tubes of distilled water.
4 Fermentation tubes of glucose bouillon.
4 Fermentation tubes of lactose bouillon.
4 Fermentation tubes of saccharose bouillon.
10 small test tubes of Dunham's solution.

A tube of each of these media will constitute a "set of media" as subsequently used for convenience in these directions.

§ 91. **Dunham's peptone solution.** This is simply a solution of peptone and sodium chloride in distilled water. The formula is as follows :

Dried peptone	1 gram.
Sodium chloride	0.5 gram.
Distilled water	100 c.c.

Dissolve the peptone and salt in the water and distribute it in the tubes (7 c.c. each) and sterilize the same as bouillon. This is used for making the indol (cholera-red) test. A sugar free bouillon has been found better but for an elementary course Dunham's solution is more desirable. See article by T. Smith, Journal of Experimental Medicine, Vol. II (1897), p. 543.

EXERCISE XXVII.

STUDY OF CERTAIN PYOGENIC BACTERIA.

§ 92. **Explanatory note.** There are a number of bacteria which are able to cause suppuration but ordinarily the formation of pus is due to the presence of streptococci and micrococci. As it is impossible to study more than a very few of these species two of the most common and one more rarely (*Bacillus pyocyaneus*) encountered organism in suppurating wounds and abscesses are chosen.

§ 93. **General directions.** Inoculate a "set of media" from each of the cultures of the following bacteria which will be furnished. *Streptococcus pyogenes*, *Micrococcus pyogenes aureus*, and *Bacillus pyocyaneus* (bacillus of green pus).

Read carefully the chapter on pyogenic bacteria in the text-book.

EXERCISE XXVIII.

PYOGENIC BACTERIA (continued).

§ 94. **General directions.** Examine and carefully describe the cultures made in Exercise XXVII. Note especially the growth on the agar, gelatin, potato, and in the fermentation tubes. In describing the color use color charts which are in the laboratory.

Examine microscopically in (1) hanging drop, and (2) stained (alkaline-methylene-blue) cover-glass preparations the bacteria from each of the bouillon and agar cultures.

Measure a few of the bacteria in the stained preparations from the agar cultures and make a drawing of them, magnified 1000 diameters.

Inoculate for Exercise XXIX., a set of media with *B. coli communis* from a culture furnished.

For suggestions in studying cultures and microscopic preparations of bacteria, see Exercise VI. and XII.

§ 95. **Making drawings of bacteria with a definite magnification.** In measuring the bacteria (§ 64) we obtain the dimensions in microns or in units of 1/1000 of a millimeter. In making a drawing, therefore, showing them magnified 1000 diameters it is simply necessary to represent each micron by one millimeter. Thus, if the organism is 2.5 μ in length and 1 μ broad, the drawing should be 2.5 mm. long and 1 mm. broad. If the drawing is to represent the organism magnified 500 diameters then each micron should be represented by 0.5 mm. For this purpose a metric rule and a pair of dividers are necessary.

EXERCISE XXIX.

BACILLUS COLI COMMUNIS.

§ 96. **Explanatory note.** Of the bacteria normally present on the mucous membranes of the animal body the colon group is, on account of its close morphological relationship to the bacilli of typhoid fever and hog cholera, of more than ordinary interest. There are varieties of this organism which approximate very closely in their biochemic properties as well as in their morphology the typhoid and also the hog-cholera bacilli. It is important that this existing variation is recognized and that the list of properties which characterize *B. coli communis* should be clearly determined. The differentiation of the colon and typhoid bacilli, as they exist in nature, is one of the difficult problems in practical bacteriological work. The culture assigned approaches very closely to the typical species.

Read as far as possible the chapters on this organism in the text-books. Also article by T. Smith, The Am. Jour. of Med. Sci., Sept., 1896, and by Adelaide W. Peckham, Journal of Exp. Med., Vol. II., (1897) p. 549.

§ 97. **General directions.** Describe the appearance of each of the cultures of *B. coli communis* made in Exercise XXVIII.

Examine the bacteria in a hanging drop preparation from the bouillon and glucose bouillon cultures.

Make and stain with carbol fuchsin a cover-glass preparation from the agar culture. Measure a few of the bacilli and record their size in the notes.

Note especially the quantity of gas formed in each of the fermentation tubes. These cultures should be kept until the next exercise when they should be examined again. If the gas formation is then completed, determine the quantity of gas and the ratio of the CO_2 to the H in the gas in each tube.

Make two gelatin plates from the bouillon culture. In making these plates use a tube of sterilized distilled water for the first dilution.

Test the culture in Dunham's solution for the presence of indol.

§ 98. **The indol test.** Add 1 c.c. of a .01% solution (fresh) of potassium nitrite and a few drops of concentrated sulphuric acid to a culture in Dunham's solution or sugar free bouillon. A pinkish color indicates the presence of indol. It is sometimes necessary to wait several hours for a reaction. In an old (3 to 5 day) culture the reaction is usually stronger than in a more recently made one.

EXERCISE XXX.

BACILLUS COLI COMMUNIS (continued).

§ 99. **General Directions.** Re-examine the cultures of *B. coli communis* and note any changes which have occurred in their appearance. Determine the gas formula in the fermentation tubes of the different sugars.

Place the milk and litmus milk cultures in the incubator and keep them there for six weeks, noting the changes which occur from week to week.

Examine and describe fully the colonies on the gelatin plates. Preserve the plates and examine them at the following two exercises and note any changes which may have occurred.

Examine microscopically, in a stained preparation, the bacteria from a colony on the gelatin plate. Preserve a preparation to accompany the notes.

Isolate *B. coli communis* from the intestine of an animal. The intestine will be furnished.

Inoculate, for Exercise XXXI a set of media with *B. cholerae suis* and one with *Bacillus typhosus* from the cultures furnished.

§ 100. **Isolating B. coli communis.** Carefully open the intestine by a longitudinal incision. Scrape away the contents, if any, from a small area of the mucous membrane. Take a loopful of the mucous from the surface of the mucous membrane and inoculate a large tube of liquefied gelatin with it. After shaking the tube carefully inoculate a second tube with two loopfuls from the first, and a third with three loopfuls from the second. Pour the gelatin into Petri dishes and label them. These plates should be examined daily. The colonies of *B. coli communis* can be distinguished from others which may appear by their thin spread-

ing growth, sharply defined but irregular borders and their bluish appearance, especially with transmitted light. Compare with colonies on gelatin plates made in Exercise XXIX.

EXERCISE XXXI.

BACILLUS CHOLERAE SUIS AND BACILLUS TYPHOSUS.

§ 101. **Explanatory note.** The bacilli of typhoid fever and of hog cholera resemble each other very closely morphologically and in certain of their cultural characters and biochemic properties. Like *B. coli communis* each of these organisms has several varieties. Already several distinct varieties of the hog-cholera bacillus have been described. (The hog-cholera group of Bacteria. Bulletin No. 6, U. S. Bureau of Animal Industry, p. 9.) Certain of the varieties of these species approach each other very closely while others approach *B. coli communis* in their various manifestations. It is important, therefore, that the morphology and properties of each of these species, should be carefully determined. The fact should be kept clearly in mind that while these two species and the colon bacillus resemble each other in certain directions they are so far as has yet been demonstrated, distinct species. The special methods of differentiation must be omitted from this elementary course. Read carefully the chapter on *Bacillus typhosus* in the textbook.

§ 102. **General directions.** Examine the plate cultures made from the intestine for the colon bacillus.

Determine the approximate number of colonies on each plate and note especially the number of colonies of *B. coli communis* and describe their appearance.

Inoculate a tube of agar, one of milk, and a fermentation tube of glucose bouillon from one of the colonies. Study

these cultures in the next exercise and compare them with the notes on cultures of *B. coli communis* in these media.

Examine and carefully describe the cultures of *B. cholerae suis*, and *B. typhosus*. Note especially the reaction of the cultures in the fermentation tubes. Examine the bouillon cultures microscopically, (a) in hanging-drop, and (b) in stained (methylene-blue) cover-glass preparations. Describe the appearance of the bacteria.

Make a series of three gelatin plate cultures from the bouillon culture of each organism.

EXERCISE XXXII.

BACILLUS CHOLERAE SUIS AND BACILLUS TYPHOSUS (continued).

§ 103. **General directions.** Re-examine all of the cultures of *B. choleræ suis* and *B. typhosus*. Note especially the condition of the fermentation tubes. Keep the milk and the litmus milk cultures in the incubator for about 5 weeks and note any changes which may take place from week to week.

Examine and carefully describe the colonies on the gelatin plates.

Try the indol test (§ 98) with the cultures in Dunham's solution.

Make and stain with alkaline methylene-blue a few (3 or 4) cover-glass preparations from the organs (liver, spleen, kidney or blood) of a rabbit which has died from the effect of the inoculation with *hog-cholera bacilli*. Note the number (few or many) of the bacteria in the preparations and preserve one of them to accompany the notes. Make a drawing of a few bacilli.

Examine and complete the notes on the culture of *B. coli communis*. Compare them with the cultures of hog-cholera and typhoid bacteria.

§ 104. **Making cover-glass preparations from tissues.** With a pair of fine forceps take up a bit of tissue from the freshly cut liver, spleen or kidney and rub it gently over the surface of a clean cover-glass. Care must be taken that the film of tissue left on the cover-glass is thin. Allow this to dry in the air after which pass them three times through the flame to fix the film to the glass. They can be stained the same as the cover-glass preparations from the cultures. These are often spoken of as smear preparations.

In making these preparations from blood, hold a cover-glass by the edge with a pair of dissecting forceps. Place a drop of blood with the platinum loop on the cover-glass near the forceps. Take a thick, square cover-glass by the edge, rest it on the first above the drop of blood, hold it at an angle of about 20° from it and draw it down over the first thus spreading the blood in a very thin even film over the surface. If the film is thick the preparation should be rejected and another one made.

EXERCISE XXXIII.

BACILLUS CHOLERAE SUIS AND BACILLUS TYPHOSUS (continued).

§ 105. Re-examine and complete the notes on all of the cultures except the milk and litmus milk which should be kept for 3 weeks longer. Carefully observe the reaction of all the liquid cultures.

Stain the flagella on the bacteria from the agar cultures (§ 72).

Compare the colonies on the gelatin plates with those of *B. coli communis.*

Make a careful comparison, in tabulated form, of the morphology, including measurements, of the bacilli them-

selves and of the appearance of the growth in the different cultures of *B. coli communis* and the bacilli of hog cholera and typhoid fever.

The cultures, excepting those in milk, can be rejected now or, if desired, they may be kept for further study and comparison.

EXERCISE XXXIV.

WIDAL SERUM TEST.

§ 106. **Explanatory note.** This test depends upon the fact that when the blood serum of a person suffering with typhoid fever or who has recently recovered from it is added to a bouillon culture of the bacillus, the bacilli become less motile and soon agglutinate in small clumps. The dilutions used vary from equal parts of serum and culture to dilutions of 1 to 50,000. It is recommended that weaker dilutions shall be used, *i. e.*, from 1:10 to 1:50. The test is believed by many to be possessed of much diagnostic value. It was observed by Dr. C. F. Dawson to apply to hog-cholera bacilli. (New York Medical Journal, Feb. 20, 1897.)

§ 107. **General directions.** Take 2 loopfuls of a fresh bouillon culture of typhoid bacilli (which will be furnished) and place them on a cover-glass, add one loopful of blood serum from a typhoid patient or the blood of an immune guinea pig and immediately make and examine a hanging-drop preparation with a loopful of the mixture. Note the effect on the motility and the aggregation into clumps. Specify the time elapsing before the agglutination is well marked.

Make a similar examination of a culture to which 1/10 blood serum has been added.

Repeat the above test with the blood from animals affected with or immunized against hog cholera.

Examine a dried specimen of blood for this reaction. Add a drop of bouillon to the drop of dried blood on a slide and after it has become well mixed add a loopful of it to a similar quantity of a fresh bouillon culture and examine it immediately in a hanging-drop.

§ 108. **Securing blood for the Widal test.** (1) Dried preparations. From a prick in the finger or lobe of the ear (if a lower animal the shaved ear is a good place) sufficiently deep to procure a drop of blood which is placed on a slide by means of a platinum loop, and allow it to dry. (2) Serum. From a similar but deeper prick or by drawing a few drops of blood from a vein with a hypodermic syringe secure a few drops of blood, place them in the bottom of a small, short sterile tube and allow the serum to ooze out. This can often be helped by separating the blood from the tube by means of a sterile wire. If retained for any length of time before making the test the serum must be kept in a cool place.

EXERCISE XXXV.

BACTERIUM (BACILLUS) SEPTICAEMIAE HEMORRHAGICAE AND MICROCOCCUS LANCEOLATUS.

§ 109. **Explanatory note.** These organisms are the causes of swine plague or infectious pneumonia in swine and of croupous or lobar pneumonia in man.

The name *Bacillus septicaemiae hemorrhagicae* was given by Hüppe to the bacillus of swine plague (Smith). This bacterium is morphologically and in its cultural characters not distinguishable from the bacillus of rabbit septicaemia, (Koch), bacillus of fowl cholera, (Pasteur), and of Schweineseuche, (Schütz). It is similar to a species of pathogenic bacteria found more or less frequently in the upper air passages of nearly all of the domesticated animals. It is very

similar also to a pathogenic bacillus found in broncho pneumonia in cattle and an infectious pneumonia in sheep. See Report on Swine Plague by T. Smith, U. S. Bureau of Animal Industry, 1891.

Micrococcus lanceolatus is the specific organism of lobar pneumonia in man. It is found in the pneumonic lung tissue and also in the saliva of a certain number of people. For the history and long synonomy of this organism see article by Prof. Welch in the Johns Hopkins Hospital Bulletin, Vol. III, p. 125. This organism resembles in many of its properties very closely that of swine plague. In studying the two species together there will be good opportnnity of comparing them and detecting the differences and similarities existing between them.

§ 110. **General directions.** From the cultures furnished, inoculate a set of media, and make a hanging drop and a cover-glass preparation from each.

Examine carefully the hanging drop preparations and describe the appearance of the bacteria.

Stain the cover-glass preparation with an aqueous solution of methyl-violet, and carefully examine and describe the bacteria. Measure a few of them with the filar micrometer and make a drawing of a few organisms magnified 1000 diameters.

Read as far as possible the articles cited on these organisms.

EXERCISE XXXVI.

BACTERIUM (BACILLUS) SEPTICAEMAE HEMORRHAGICAE AND MICROCOCCUS LANCEOLATUS (continued.)

§ 111. **General directions.** Carefully examine and describe the cultures made in Exercise XXXV.

Examine the agar and bouillon cultures microscopically in both the living condition and in cover-glass preparations

stained with alkaline methylene-blue, carbol fuchsin and an aqueous solution of methyl-violet.

Describe the appearance of the bacteria and make a drawing of a few of them from one preparation.

Preserve a preparation of each species to accompany notes.

If there is any growth in the gelatin tube make a series of 3 gelatin plates from the bouillon culture.

EXERCISE XXXVII.

BACTERIUM (BACILLUS) SEPTICAEMIAE HEMORRHAGICAE AND MICROCOCCUS LANCEOLATUS (continued).

§ 112. **General directions.** Re-examine all the cultures of these bacteria paying special attention to the reactions of the liquid cultures.

Make the indol test with the cultures in Dunham's solution.

Make, stain, and examine a cover-glass preparation from an organ or the blood of a rabbit which has died from the inoculation with swine plague bacteria, and also a preparation made from a rabbit which has died from the inoculation with *Micrococcus lanceolatus*. Stain the preparations with an aqueous solution of fuchsin. Study the bacteria in these preparations and carefully compare the two. Indicate in the notes the differences, if any are found.

Keep the cultures until the next exercise and compare them again, after which they may be rejected.

EXERCISE XXXVIII.

BACTERIUM (BACILLUS) OF TUBERCULOSIS.

§ 113. **Explanatory note.** The tubercle bacterium does not grow readily on the ordinary media. For its cultivation blood serum, glycerine agar, or bouillon containing 5 to 7% glycerine are ordinarily used. It is with much difficulty that it is made to grow from lesions in tuberculous animals, but when a culture is once started it can, on the media mentioned above, and sometimes on agar, be cultivated in subcultures with comparative ease. It grows very slowly and it is necessary that the temperature should be kept, without variation, at about 37° C. On account of these difficulties it is not practicable in a general course to cultivate this organism, but cultures on solid and liquid media will be furnished by the instructor for examination. It is important, however, to be able to recognize this organism in tissues and sputum and consequently the following additional exercise in staining and studying it is given.

§ 114. **General directions.** Examine and carefully describe the appearance of the cultures of the tubercle bacterium on glycerine agar, and in glycerine bouillon, furnished.

Make cover-glass preparations from the culture furnished for that purpose, and stain.

Make 4 cover-glass preparations from tuberculous sputum and stain for tubercle bacteria. It is often desirable to counter stain the specimens from sputa. Stain 2 of them by Gabbett's method, and 2 with carbol fuchsin and decolorize without counter staining. Make a few (2 or 3) cover-glass preparations from the liver or spleen of a guinea pig, which has died from tuberculosis and stain them for tubercle bacteria. Stain one with carbol fuchsin and decolorize with sulphuric acid, and stain one by Gabbett's method, and counter-stain.

Measure the tubercle bacteria in one of the preparations and make a drawing showing a few of them magnified 1000 diameters.

§ 115. **Making cover-glass preparations from sputum.** Select the little yellowish colored masses, if present, remove them by means of the fine forceps or platinum loop, and spread them on the cover-glass in a thin layer. If the sputum is homogeneous make the preparations the same as from cultures, using a small loopful of the liquid. If the sputum is viscid it is necessary to use the forceps to spread the film on the cover-glass. After drying, the films are fixed by passing the preparations through the flame, after which they are ready to be stained.

§ 116. **Gabbett's method of staining tubercle bacteria.**

(1) The stain (carbol fuchsin) :

Fuchsin, ---------- 1 gram.
Absolute alchool, ---------- 10 c. c.
5% carbolic acid ---------- 100 c. c.

(2) The decolorizer and counter stain :

Methylene-blue powder ---------- 2 grams.
10% sulphuric acid, ---------- 100 c. c.

Stain the preparation with the first solution as described in (§ 76) then rinse in water and stain one minute with the second solution which decolorizes and counter stains at the same time, and again rinse in water. If the film has a bluish tint it is ready for examination, if not, it should be stained a little longer in the second solution. In these preparations the tubercle bacteria should appear as slender, more or less curved, rod-shaped bodies of a deep reddish color while the surrounding tissue and other bacteria present are stained a more or less intense blue.

EXERCISE XXXIX.

BACTERIUM (BACILLUS) MALLEI.

§ 117. **Explanatory note.** This organism grows most characteristically on potato, and somewhat feebly in the other media heretofore used. It develops readily on acid agar, and in acid glycerine agar and acid glycerine bouillon. For this reason it is not inoculated into the full set of media. In diagnosing glanders, it is customary to inoculate guinea-pigs with the suspected material. From the lesions in these animals, if the disease develops, pure cultures can usually be obtained. It can be identified from its morphologic and cultural characters.

§ 118. **General directions.** Inoculate a tube of potato, one of agar, one of acid agar, one of acid glycerine agar, one of glucose agar and one of bouillon from a culture furnished. (The special media here introduced will be furnished by the instructor).

§ 119. **The preparation of acid agar.** This is prepared the same as ordinary agar (§ 16) with the omission of the sodium hydrate in the bouillon from which it is made.

§ 120. **The preparation of acid glycerine agar.** Add 5% glycerine to acid agar before sterilizing it.

EXERCISE XL.

BACTERIUM (BACILLUS) MALLEI, (continued).

§ 121. **General directions.** Examine and carefully describe all the cultures of *Bacterium mallei.*

Make three cover-glass preparations from the acid agar and from the bouillon cultures, and stain one of each with alkaline methylene-blue, one with carbol fuchsin and one

with an aqueous solution of methyl-violet. Describe the bacteria and make a drawing of a few of them. Preserve one preparation. Keep the cultures and re-examine them at each of the three following exercises. Note especially the character and color of the growth on the potato.

EXERCISE XLI.

ACTINOMYCOSIS.

§ 122. **Explanatory note.** Although actinomycosis is not a bacterial disease, it is of peculiar interest as it is caused by a fungus (ray fungus) of higher rank than bacteria. It occurs in man and certain of the lower animals. The ray fungus can be cultivated on glycerine agar and certain other media but ordinarily it can be easily detected in fresh or stained preparations of the affected tissue.

§ 123. **General directions.** Examine a culture of actinomycosis furnished and describe its appearance. Make a hanging drop and a stained cover-glass preparation from it. Examine and make a drawing of the fungus.

Examine sections (which will be furnished) of animal tissues containing the ray-fungus and also permanent preparations of the fungus isolated by maceration from actinomycotic tissue.

Inoculate a set of media with *Bacterium anthracis* for studying at the next exercise.

EXERCISE XLII.

BACTERIUM (BACILLUS) ANTHRACIS.

§ 124. **General directions.** Examine and describe each of the cultures of this organism inoculated in the last Exercise.

Examine microscopically the bouillon and agar cultures in both hanging drop and stained cover-glass preparations. Stain a preparation with each of the three ordinary staining solutions heretofore used.

Measure a few of the bacteria in a stained preparation and make a drawing of them magnified 1000 diameters.

Make a series of 3 agar plates from the bouillon culture.

Examine sections of animal tissue containing anthrax bacteria. Make and examine a few cover-glass preparations from the liver of an animal (guinea-pig or rabbit) which has just died of anthrax. (This will be furnished by the instructor).

EXERCISE XLIII.

BACTERIUM (BACILLUS) ANTHRACIS (continued).

§ 125. **General directions.** Re-examine all of the cultures of Bacterium anthracis and describe any changes in their appearance which may have taken place.

Examine the agar culture for spores in (1) a hanging-drop preparation, and (2) a stained cover-glass preparation. Describe the appearance of the bacteria and spores in a preparation from each.

Stain the spores (Exercise XVII.)

Study and describe the appearance of the colonies on the agar plates. Make an outline drawing of a few of the surface and deep colonies.

Reject all cultures except the agar plates which may be kept until the next exercise for further observation before rejecting. (These cultures should be put in charge of the instructor who will see that the spores are destroyed before the tubes are cleaned).

Inoculate a set of media with *Bacterium diphtheriae*, for study at the next exercise.

EXERCISE XLIV.

BACTERIUM (BACILLUS) DIPHTHERIAE.

§ 126. **Explanatory note.** The bacterium of diphtheria is often called the Klebs-Loeffler bacillus. It is the specific cause of diphtheria in man but it is not, so far as known, the cause of diphtheria in pigeons and poultry. It is found in the throat of people suffering with diphtheria, but it is not found ordinarily elsewhere in the body. Its appearance in the throat lesions is availed of in diagnosing the disease. For this reason it is especially important that its morphology as well as its cultural characters should be carefully determined. Although this organism grows on nearly all of the media commonly used, its development is more rapid and its growth more characteristic on Loeffler's blood serum. The bacterium of diphtheria seems to be modified in its morphology in growing on different media more than any of the other pathogenic bacteria. Particular attention should be given to its morphology and staining properties.

§ 127. **General Directions.** Examine and describe the cultures made in Exercise XLIII.

Examine the agar and buillon cultures microscopically in (1) hanging-drop preparations, and (2) stained cover-glass preparations. Stain with alkaline methylene-blue and with carbol fuchsin. Also stain a preparation after Gram's method.

Inoculate a tube of glycerine agar, one of blood serum, and one of Loeffler's blood serum from the agar culture.

The blood serum and Loeffler's serum necessary for this exercise will be furnished.

§ 128. **The preparation of blood serum.** When a small quantity is sufficient it can be obtained from a dog aseptically. The animal is properly tied on the operating table, etherized, the skin over the carotid or femoral artery

is thoroughly disinfected and turned back, the artery exposed, a sterile glass canula inserted and the blood collected, by means of a sterile rubber tube attached to the canula, in a sterile flask. After the serum is formed it can be drawn off with a sterile pipette, and distributed in small sterile test-tubes (5—7 c.c. in each). It is well to set the liquid serum in an incubator for a few days to test its sterility. The tubes of liquid serum are inclined (the same as agar) and placed in a blood serum sterilizer, or other chamber in which the temperature can be raised to 70° or 75° C., and kept there until the serum has set. Label and store.

If larger quantities of the blood are required, it is more convenient to collect it from the bleeding animals (cattle or sheep) in a slaughter house. It is often necessary to sterilize the liquid serum after it has been distributed in tubes (5 c.c. in each), when it has been collected in this manner. This can be done by heating them in a water bath at 62° C. for 2 hours each day for four consecutive days.

§ 129. **The preparation of Loeffler's blood serum.** This consists of neutral bouillon (prepared from meat) containing 1% glucose, 1 part, liquid blood serum 3 parts. Mix and distribute in sterile test tubes, incline and solidify the same as blood serum. The temperature should be about 75° C. and the exposure will be necessarily longer than for the pure blood serum. Label and store.

For other methods of preparing serum and other special media. See text-books.

§ 130. **Staining bacteria by Gram's method.** Prepare the cover-glass preparations as already described. Stain them in gentian-violet aniline water or in a saturated alcoholic solution of gentian violet in 5% carbolic acid in the proportion of 1 to 20 for from 5 to 7 minutes. Rinse in water and transfer them to a watch glass containing Gram's solution until the color becomes quite black. This requires from one to two minutes; then place the preparations in a

watch glass containing alcohol and allow them to remain there until the color has almost entirely disappeared, or has become a pale gray. Rinse in water and examine at once, or allow them to dry and mount in balsam. (Sections of tissues must be dehydrated and cleared before mounting.)

Formula for Gram's solution (Lugal's):

Iodine	1 gram.
Potassium iodide	2 grams.
Distilled water	300 c. c.

Certain bacteria stain deeply when treated by this method while others are decolorized by the alcohol. On this account it is of some differential value.

EXERCISE XLV.

BACTERIUM (BACILLUS) DIPHTHERIAE (continued).

§ 131. **General Directions.** Re examine all the cultures of *B. diphtheriae*, and describe all changes which have appeared in them.

Examine microscopically, in stained cover-glass preparations, the bacteria from the glycerine agar, blood serum, and Loeffler's blood serum cultures. Stain with alkaline methylene-blue, and note especially the way the bacteria stain. Stain a few preparations with the other staining solutions and compare with the methylene-blue stain.

Note with special care the morphology of the bacteria and make a drawing of a few of them.

Examine sections of diphtheritic membrane showing diphtheria bacteria, and streptococci (furnished). Reject all cultures of diphtheria bacteria.

Inoculate from the unknown cultures furnished such media as the requirements of the next exercise seem to demand.

EXERCISE XLVI.

DETERMINE THE SPECIES OF BACTERIA.

§ 132. **Explanatory note.** The two cultures of bacteria assigned belong to species already studied. The student should identify the species of the bacteria in the cultures. To do this such media should be inoculated and such microscopic examinations made as he thinks necessary. The notes should contain a complete record of the work and the reasons for the identifications made.

§ 133. **General directions.** Identify the bacteria in the cultures assigned at the last exercise.

EXERCISE XLVII.

ISOLATING AND IDENTIFYING BACTERIA FROM ANIMAL TISSUES.

§ 134. **Explanatory note.** In making a bacteriological investigation into the cause of death in an animal or man it is necessary to make cultures from the various organs and the blood to find whether or not there are any pathogenic or other bacteria present. This necessitates a knowledge of making cultures from animal tissues. In this exercise an experimental animal (rabbit or guinea-pig) will be provided which has died from some bacterial disease. The purpose of this examination is to find out what that disease is. To save animals each student will make cultures from but one organ. Opportunity will be afforded from time to time during the course for making cultures from various animal tissues.

§ 135. **General directions.** The experimental animal will be furnished tied out on a post-mortem tray and the

viscera exposed. (Directions for the post-mortem examination will be given in the course in pathology).

Inoculate a tube of bouillon, one of agar, and a fermentation tube of glucose bouillon, from either the liver, spleen, or kidney. (In an actual investigation of an unknown disease, cultures should be made from all of the organs, blood, and lymphatic glands).

Make a series of 3 agar plate cultures.

Make several cover-glass preparations from the organ from which the cultures were made.

Stain and examine the cover-glass preparations and describe the bacteria, if any are found. Stain with alkaline methylene-blue and carbol fuchsin. (It is sometimes necessary to preserve pieces of the tissue in alcohol or to fix them in some of the other fixing fluids for sectioning and staining, preparatory to studying them).

Preserve one or more of the cover-glass preparations to accompany the notes.

§ 136. **Making cultures from animal tissues.** Heat a platinum spatula to a red heat in a gas flame and scorch the surface of the organ. Flame a pair of fine forceps and tear the scorched surface away, and with the platinum loop take up a loopful of the tissue underneath with which inoculate the media. It is also desirable to inoculate a tube of slant agar with the needle by drawing it over the surface after changing it with tissue. In making plate cultures use a loopful of the crushed tissue for the first tube. The quantity of the tissue necessary to give a desired number of colonies cannot be anticipated, although experience in working with different organisms in animals renders one able to approximate the amount required.

EXERCISE XLVIII.

ISOLATING AND IDENTIFYING BACTERIA FROM ANIMAL TISSUES (Continued).

§ 137. **General Directions.** Examine and describe all of the cultures made from the animal tissues.

Examine the bouillon and agar cultures microscopically in the fresh condition and in stained cover-glass preparations.

If the species can not be determined from these cultures and examinations, make such others from the agar culture as may be necessary to do so. Examine these at the next exercise when the notes can be completed.

State in the notes the facts upon which the identification is based.

EXERCISE XLIX.

THE EXAMINATION OF SECTIONS OF TISSUE CONTAINING BACTERIA.

§ 138. **Explanatory note.** The preparation of tissues for sectioning and the study of the tissue changes more properly belong in the course in pathology. It is important, however, that one is able to distinguish bacteria in the lesions which they produce. For this reason an exercise is devoted to the study of bacteria in sections of tissues already stained and mounted. These include the various pneumonias, tuberculosis, anthrax, hog cholera, typhoid, septicaemia, etc.

§ 139. **General directions.** Examine the sections furnished for the bacteria and note especially their distribution in the tissues. Make drawings of a few of the bacteria from each preparation.

EXERCISE L.

BACTERIOLOGICAL EXAMINATION OF PUS AND EXUDATES.

§ 140. **Explanatory note.** It is often very desirable for diagnostic purposes to make a bacteriological examination of the pus from abcesses and the muco-purulent discharges or exudates from mucous or serous membranes.

Several diseases can be diagnosed in this way. It is often necessary to make cultures and it is always advisable to do so whenever the material is in a suitable condition. Among the specific diseases for which such an examination is especially valuable are actinomycosis, gonorrhea, diphtheria, and tuberculosis. It is often desirable to determine the character of the bacteria in the numerous abcesses and suppurating wounds encountered in both man and the lower animals. These examinations will be made from the more desirable cases as they appear from time to time. In this exercise such cover-glass preparations will be examined as have been accumulated for this purpose.

§ 141. **General directions.** Examine the pus in the fresh condition and note its composition, leucocytes, red blood corpusles, fungi (actinomycosis), etc.

Make cover-glass preparations and stain one or more of them with carbol fuchsin and one with alkaline methylene-blue and examine. Note the celluar tissue elements present and also describe the bacteria found. If the pus is from a case suspected to be of a specific nature stain and examine for the corresponding organism.

If actinomycosis, the ray fungus may be seen better in the fresh preparation. Add a drop of a 10% solution of caustic potash to a loopful of pus on the slide and cover with a cover-glass and examine.

If gonorrheal discharge, stain the cover-glass preparations with alkaline methylene-blue or with carbol fuchsin. Note

the appearance of the cocci both within and outside of the pus cells.

If from supposed tuberculosis, stain for that organism.

If from diphtheria, stain for that organism and note the morphology of the bacteria.

If from the pus of an abscess, stain for pyogenic bacteria.

§ 142. **Making cover-glass preparations from pus.** Spread as thin a film of the pus as possible on the cover-glass. This can be readily done by drawing the edge of a square cover-glass over the surface of another cover-glass on which a bit of the pus has been placed. See method for making cover-glass preparations from blood (§ 104).

EXERCISE LI.

A BACTERIOLOGICAL EXAMINATION OF THE SKIN FOR MICROCOCCUS (STAPHYLOCOCCUS) EPIDERMIDIS ALBUS.

§ 143. **Explanatory note.** There is liable to be on or in the skin a number of bacteria which resist the ordinary methods of cleansing, owing to their being deep seated in the epidermis. The most important among these is *M.* (*Staph.*) *epidermidis albus*. These organisms often infect wounds in surgical operations. An abrasion of the skin with a sterile instrument may be followed by the infection of the wound with this or other species of bacteria which were on, or in the skin itself. The work of this exercise is to demonstrate the presence of these organisms.

§ 144. **General directions.** Wash the hands thoroughly with soap and water, using the brush. Then wash them in a solution of carbonate of sodium and rinse thoroughly in boiled water and wipe with a sterilized towel.

With a flamed and cooled scapel scrape the epidermis over a small area about the finger nails and with these scrapings

inoculate a tube of bouillon and make a series of 2 agar plate cultures.

At the next exercise describe these cultures and examine the colonies microscopically to determine the genera of bacteria. If a micrococcus which grows in clumps is found, inoculate a tube of agar with it and at the following exercise examine and describe its appearance. Indicate in the notes the number of colonies of bacteria which developed in the plate cultures and the genera which appear in the bouillon culture.

EXERCISE LII.

DETERMINE THE THERMAL DEATH POINT OF BACTERIA.

§ 145. **Explanatory note.** It is important to know the minimum temperature which will kill bacteria, especially the pathogenic forms. The uses for such knowledge are numerous in practical disinfection and pasteurization. For the various methods employed in making these determinations, see text books and special articles on this subject. The method here given and which can be followed by a full section of students will give only approximate results. It should not vary, however, more than one degree from the actual thermal death point in moist heat.

§ 146. **General directions.** Inoculate 5 tubes of bouillon from each of two cultures (*B. subtilis*, old culture, and *B. typhosus*) furnished.

In inoculating be sure not to touch the sides of the tube with the inoculating loop. The tubes should have stood in the water bath at 60° C. for at least 10 minutes before they are inoculated.

Place one of these tubes in the incubator for a control. Stand the others in a wire basket and set them in the thermoregulated water bath which is at 60° C. The water should

come just above the liquid in the tubes. Remove the tubes, one of each species, as follows. One in 5 minutes, one in 10 minutes, one in 15 minutes and one in 20 minutes. Label and place them in the incubator.

At the next exercise, examine the heated tubes and note which are clear and which contain a growth. If the tubes heated for 10 minutes or longer have a growth, repeat the experiment at 70° C. If this fails to destroy them repeat at 80° C. and if necessary apply a still higher temperature.

Examine the cultures microscopically in all the fertile tubes to determine if they are pure.

Explain the cause for the difference in the thermal death point between the two organisms.

Inoculate for the next exercise a tube of bouillon from each of the cultures of *M.* (*Staph.*) *pyogenes aureus* and *B. subtilis* furnished.

EXERCISE LIII.

DETERMINING THE EFFICIENCY OF DISINFECTANTS.

§ 147. **Explanatory note.** The efficiency of the more commonly used disinfectants has been determined for most of the pathogenic bacteria, but new disinfectants are constantly being put upon the market, and before it is safe to use or recommend them, their efficiency should be determined. With many of the disinfectants, such as carbolic acid, corrosive-sublimate, lime, and the mineral acids, much stronger solutions are commonly used than are actually necessary to kill the bacteria, owing to the fact that frequently it is necessary to allow for an indefinite waste due to the union of the disinfectant with other substances, usually organic, with which the bacteria are mixed. For the different methods of testing the efficiency of disinfectants, see text-books. A very simple process is given here.

§ 148. **General directions.** Prepare 20 c. c. of each of a .25, .10, .02% solutions of formalin (40% formaldehyde in water), and place them in sterile tubes, putting 10 c. c. in each. Use distilled water in making the dilutions. Add to each of the tubes in one set ¼ c. c. of the bouillon culture of *Micrococcus (staphy) pyogenes.* And to each of the tubes in the other set, the same quantity of the culture of *B. subtilis.*

Use a sterile pipette for adding the culture to the disinfectant.

Inoculate a tube of bouillon with six loopfuls from each of these tubes after the expiration of the following periods of time, viz. : 1 min., 5 min., 10 min., 20 min., and 30 min. In making these inoculations allow the loop to go to the bottom of the inoculated tube. Label each inoculated tube with the strength of the disinfectant and time of exposure and place it in the incubator. It should be noted that the adding of ¼ c. c. of culture diluted slightly the strength of the disinfectant.

Note at the next exercise the condition of each inoculated tube. From them the approximate strength and time for the disinfectant to destroy the bacteria can be determined. When this is found the more exact strength and time can be determined by repeating the experiment with weaker dilutions or shorter exposures or both.

EXERCISE LIV.

TESTING DISINFECTANTS (continued).

§ 149. **General directions.** Prepare 2 sets of tubes containing 10 c.c. each with 2.5, 1.0 and 0.5% solutions of carbolic acid respectively, and test their effect upon the organisms used in the last exercise and by the same method. Note the conditions of the inoculated tubes at the next exercise.

Examine the tubes inoculated during the preceding exercise.

Allow the inoculated tubes to remain in the incubator for several days and note whether or not any of them develop after the first 24 hours. If they do examine them microscopically to determine if the culture is pure. Observe in these cultures the difference between immediate destruction and the retarding of the growth of the bacteria.

EXERCISE LV.

PASTEURIZING AND STERLIZING MILK.

§ 150. **Explanatory note.** Milk is pasteurized, in the present acceptance of the term, when all of the pathogenic bacteria which it may happen to contain (with the exception of the spores of Anthrax) are destroyed with the more important Saprophytes. It is not necessarily sterile although it sometimes is. The temperature and time for heating is from 60–68° C. for 20 minutes.

In this exercise it is the purpose to study the effect of this process on the bacteria of milk and to compare its effect with that of sterilization.

§ 151. **General directions.** From the fresh milk provided, make 3 agar plates, using 1, 2 and 3 loopfuls respectively of the milk. Put 20 c.c. in a large tube and set it in the incubator. Put 25 c.c. in each of 4 large test tubes. Sterilize 2 of them by boiling for 30 min. in a closed water bath, and pasteurize the other 2 by heating them in the water bath for 30 minutes at 65° C. It requires about 10 minutes for the milk in the tubes to reach the temperature of the water, leaving the milk exposed to the temperature of the water for 20 minutes and cool.

After the tubes are cooled make 3 agar plates from one of the tubes treated by each process, using 1 loopful of milk for

the first plate, 3 loopfuls for the second, and ¼ c.c. (measure with a graduated pipette) for the third. Place one of the tubes of milk treated by each process with the plate cultures, in the incubator, and leave the other tubes with a tube of the fresh milk at the room temperature.

At the next exercise note carefully the condition of the milk in each of the various tubes, and also the number of colonies on the agar plates.

Keep the tubes of milk for further examination at the following exercise, after which they may be rejected.

EXERCISE LVI.

THE QUANTITATIVE BACTERIOLOGICAL EXAMINATION OF WATER.

§ 152. **Explanatory note.** This is to determine the number of bacteria in water. In preparing media for this purpose the directions given in the Journal of American Public Health Association for Jan., 1898, p. 60, should be followed. The conditions of temperature and of media which favor growth differ for different species. Many water bacteria will not grow at the incubator temperature while others which may be in it grow very slowly at the room temperature. To determine numbers it is better to grow the bacteria in gelatin plates at the temperature of the room. (In an actual examination a much larger number of plate cultures should be made than can be managed here.)

§ 153. **General directions.** Make from the properly collected water 4 gelatin plates using a definite quantity of water for each. It may be safe to begin with to inoculate these tubes with 0.1, 0.25, 0.50, and 1.00 c.c. respectively.

To determine if there are gas producing bacteria, and the approximate number of these if any, inoculate 10 fermentation tubes with 1 c. c. each and 5 with $\frac{1}{5}$ c. c. each. (In place of

the fermentation tubes glucose agar can be used. In this case one fermentation tube of glucose bouillon should be inoculated with 5 c. c. of the water to determine the quantity of gas produced if there is any). From the gas produced in these tubes determine approximately the number of the gas producing bacteria.

Careful and full notes should be taken on this examination the preliminary methods for making a bacteriological examination have already been given and this is largely in the nature of an investigation by each stndent. It is not expected that the special methods other than those used in the laboratory for pathogenic bacteria will be tried.

§ 154. **Collecting water.** If the water is collected from a spicket or pump allow it to flow for 2 or 3 minutes first and then collect the desired quantity, 100–200 c. c. in a sterile bottle and cork tightly, or if near at hand, absorbent cotton plugs may be used.

If from a stream or river withdraw the stopper and immerse the sterile bottle, to the depth desired and allow it to fill. There are several mechanical devices for collecting water from considerable depths from the surface.

EXERCISE LVII.

THE QUANTITATIVE EXAMINATION OF WATER (continued).

§ 155. **General directions.** Examine the cultures and count the colonies on the plates and estimate from them the number of bacteria in a cubic centimeter of the water. That is if there are 40 colonies on the plate containing .1 c.c. of water there are 400 bacteria in 1 c.c. of it.

From the cultures in the glucose media estimate the number of gas producing bacteria present.

Describe the appearance of the different colonies and indicate the approximate number of each kind.

Keep the plate cultures until the following exercise and re-examine and count the colonies.

Determine the obviously different genera of bacteria by making a microscopic examination of the different colonies.

§ 156. **Estimating the number of gas producing bacteria in water.** If there is gas in all of the ten fermentation tubes inoculated with 1 c. c. each, it would show that there were one or more of these bacteria in each cubic centimeter. If 3 of the 5 tubes inoculated with $\frac{1}{5}$ c. c. each contained gas it would indicate that there were at least 3 gas producing bacteria in one cubic centimeter. The preliminary results must be verified by repeated examinations.

EXERCISE LVIII.

THE QUALITATIVE EXAMINATION OF WATER.

§ 157. **Explanatory note.** The qualitative examination of water consists in determining the species of bacteria present. From a sanitary standpoint it consists in finding, if present, those species which may be the cause of disease among people or animals consuming it. The pathogenic bacteria which may be in the water will depend upon the conditions, but usually in this country water is examined for typhoid and hog cholera bacilli, *B. coli communis* and *B. pyocyaneus*.

In India the spirillum of Asiatic cholera may be found in the water. Occasionally, anthrax may be suspected. It should be stated that *B. fluorescens liquefaciens*, pseudo typhoid and the transitional form of the colon group are to be carefully differentiated from *B. pyocyaneus* and *B. typhosus*. Owing to imperfect descriptions many of the common soil and water bacteria cannot be readily identified. The genera of these is all that is expected here.

§ 158. **General directions.** Make at least four gelatin plate cultures, and such others as may seem necessary to

determine the different species, especially of pathogenic bacteria, and their relative number in the specimen of water furnished.

Read carefully the methods for water analysis in the text-books.

EXERCISE LIX.

THE QUALITATIVE EXAMINATION OF WATER (continued).

§ 159. **General directions.** Examine the cultures made in Exercise LVIII, and make such others (subcultures from colonies, etc.) as may seem necessary to determine the genera and species of bacteria present with the relative number of each per cubic centimeter.

EXERCISE LX.

EXAMINATION OF CERTAIN BACTERIA NOT STUDIED IN THE LABORATORY.

§ 160. **Explanatory note.** This exercise will be devoted to a review of preparations of important bacteria not studied in the laboratory but demonstrated at the time of their consideration in the lectures. Unfortunately the number necessarily omitted is large. In this review those species whose morphology forms an important part in their identification such as the bacillus of tetanus, malignant oedema, and various spirilla will be considered. Certain of the pathogenic protozoa will also be demonstrated. These will be studied more thoroughly in the course in Pathology.

§ 161. **General directions.** Examine and make drawings of the bacteria in the preparations furnished.

Complete and hand in all notes on laboratory work.

Have all apparatus for individual use inspected by the instructor.